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Ninth Technical Alfalfa Conference Proceedings

HELD AT LINCOLN, NEBRASKA,
NOVEMBER 17, 1965

UNITED STATES DEPARTMENT
OF AGRICULTURE
Agricultural Research Service

THE NINTH TECHNICAL ALFALFA CONFERENCE was held November 17, 1965, in Lincoln, Nebraska, at the Nebraska Center for Continuing Education. It was attended by forage growers and processors, equipment manufacturers, representatives of feed manufacturers, feedlot operators, laboratory technicians, and research personnel in State Agricultural Experiment Stations and the U. S. Department of Agriculture.

Dr. H. H. Kramer, Director of the Agricultural Experiment Station of the University of Nebraska, gave the assemblage a warm welcome and expressed the appreciation of the University for the interest this groups has in alfalfa, a principal crop of Nebraska.

The meeting was cosponsored by the American Dehydrators Association and the Nebraska Agricultural Experiment Station. The program was developed by George O. Kohler, Chief, Field Crops Laboratory, Western Utilization Research and Development Division, Agricultural Research Service, USDA; Richard L. Kathe, Executive Vice President, American Dehydrators Association; and William R. Kehr, Research Agronomist, Crops Research Division, Agricultural Research Service, USDA.

Staff members engaged in forage investigations reported recent researches conducted by the Western Utilization Research and Development Division with particular emphasis on the pigmenting potency of alfalfa and its determination and the method of determining xanthophylls in dehydrated alfalfa. Members of the Crops Research Division reported on varietal improvement of alfalfa and alfalfa management for dehydration. State Experiment Station workers reported on alfalfa management in the field and took part in the pigmentation-xanthophyll discussion.

Speakers and their organizations are responsible for the information they have contributed, and they should be consulted by those who may wish to reproduce their speeches, wholly or in part. Occasional mention of commercial products does not imply recommendation by the U. S. Department of Agriculture.

This report was prepared in the Western Regional Research Laboratory, Albany, California 94710--headquarters of the Western Utilization Research and Development Division of the U. S. Department of Agriculture.

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NINTH TECHNICAL ALFALFA CONFERENCE PROCEEDINGS

INTRODUCTORY REMARKS

G. O. Kohler

Western Utilization Research and Development Division, USDA
Albany, California

It falls to my lot, in addition to acting as chairman this morning, to make a few opening remarks on behalf of the U.S. Department of Agriculture's Western Utilization Research Division. As cosponsors with the American Dehydrators Association we at Albany, California, have had the pleasure of hosting this group in six of the last eight conferences. The 8th Conference was convened at Colorado Springs in 1963 in conjunction with the ADA Annual Convention and the 5th Conference was held at Colorado State University in 1958, at the time of the Summer Board Meeting of ADA in Estes Park. This 9th Conference has the good fortune to have an additional cosponsor in the Nebraska Agricultural Experiment Station. We welcome their cooperation.

We in the USDA research center on the West Coast have a Field Crops Laboratory as one of our several commodity groups. Within this laboratory is a group concerned with forage crops and within that group are individuals investigating carotenoid pigments, phenolic compounds, saponins, amino acids, cellulosic compounds, and alfalfa separation procedures. While most of the workers are full-time employees of USDA, our efforts are supplemented from time to time by foreign research workers on leave, by employees hired with funds supplied by groups such as the American Dehydrators Association, or other non-USDA groups. Such an "extra" worker in our laboratory at present is Joe Chrisman, who is an employee of the Department of Agriculture and Economic Development of the State of Nebraska and a collaborator in our Field Crops Laboratory.

Over the years, our work on alfalfa and other forage crops has occupied many hours of many people. Much of the work has been basic, on composition and methodology. However the end point is always the improved and increased practical utilization of forages. While we have the responsibility for utilization research on all forages, most of our efforts have been directed to alfalfa, since it is the most important processed forage. Our program is national in scope and is not restricted in any way to any region. Thus, the name Western Utilization Research and Development Division cannot be considered restrictive. We are particularly interested in Nebraska, since it is here that over 40% of the processed forage is produced. This is of course why we operated our pilot scale dehydrator in Cozad this year. American Dehydrators Association, particularly its Alfalfa Research Council, has been of great value in keeping alert to new problem areas.

Not only is work carried on in our laboratory but through research contracts such as the one currently in effect here at the Nebraska Station. Through cooperative agreements, memoranda of understanding, and informal cooperation, we have been able to get much forage crops research accomplished in

State Experiment Stations and other research groups both in the United States and in some foreign countries. As I mentioned, our utilization research has also benefited by having workers from other countries spend some months with us. Several years ago, Dr. J. M. A. Tilley of the Grassland Research Institute in England worked with us for 6 months. With us today is Mogens Israelsen from the Research Institute for Commercial and Industrial Plants, Kolding, Denmark, who has just concluded about five months of work under a NATO Science Fellowship in what I have dubbed "Cell X", studying the carotenoid pigment problems. The X, of course, comes from "xanthophyll".

In closing, I should like to express my appreciation to the American Dehydrators Association as a group and to Dick Kathe and the individual members who have given us excellent cooperation and moral support. Such industry support is an absolute essential for survival and development of any research program of this type.

THE ROLE OF DEHYDRATED ALFALFA IN THE RATIONS OF ANIMALS

Carrol Syverson
President, American Dehydrators Association

Alfalfa is one of several basic crops that can be grown in abundance in animal-producing areas. It has been an economical and excellent source of nutrients for all animal species and types. Its value was first recognized for ruminants and it was brought to this continent for that purpose. With the discovery of vitamins, it was discovered that it had a place in the ration of the nonruminant, and from this discovery the sun-cured hay grinding industry developed. The next advancement was artificial drying or dehydration at a predetermined stage of growth. Other advances were gas storage, pelleting, and use of antioxidants to preserve beta-carotene and xanthophyll.

For 20 years, the American Dehydrators Association has financed research by grants-in-aid to get basic information on the nutrient content of its products. During 1964 a great advance was made when it financed an assay project to determine on representative commercial grades, by the most accurate and reliable methods, the nutrient content, such as amino acids, minerals and vitamins. Dehydrated alfalfa is the only feed ingredient on which such reliable data are available. Now the industry is starting to get information on the production and feeding of animals. There has been a transition from many small producing units operated by general farmers to larger producing units operated by a business corporation. To serve this new pattern of animal production, it is essential to learn what is needed in the rations of animals produced under large commercial conditions. This needs to be followed with a program of research to learn what type of dehydrated alfalfa is most economical in the future least-cost rations.

Dehydrated alfalfa has been regarded as a source of vitamins and some essential reproduction factors. In the future, it will also serve as a desirable, essential and quality controlled source of nutritious forage for ruminants

and some monogastrics. In the future, our industry will be alert and sensitive to the needs of the rations fed to animals to produce constant quality animal products at least cost.

VARIETAL IMPROVEMENT OF ALFALFA AS RELATED TO PRODUCT QUALITY AND UTILITY

C. H. Hanson

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There is no simple definition for forage quality. To obtain one, I refer you to the nutritionists. My report deals primarily with certain biochemical differences among alfalfa varieties affecting quality, and the prospects of modifying them by breeding and selection. Breeding alfalfa to improve quality is in its infancy; the potentials have not been determined. As I develop this topic, please keep in mind that crops management practices, such as time of cutting, are extremely important variables. The management variable will be discussed by Dr. Dale Smith.

Insect resistance. H. W. Johnson (Phytopathology 26: 1061-63, 1936) showed that alfalfa yellowed by the potato leafhopper contained much less carotene than did alfalfa of the same variety protected from the leafhopper. These results were later confirmed by W. E. Ham and H. M. Tysdal (J. Am. Soc. Agron. 38: 68-74, 1946). The latter also found that strains resistant to the potato leafhopper had higher carotene content than did susceptible strains. W. R. Kehr, Lincoln, Nebraska, and C. P. Wilsie, Ames, Iowa, made similar observations (letter communications). It is evident that development of varieties resistant to leafhopper yellowing would be effective in producing higher carotene hay for areas subject to attack by this insect.

Disease resistance. R. D. Brigham (Agron. J. 51: 365, 1959) observed a sharp drop in crude protein content in alfalfa leaves infected with Cercospora leafspot. Leaves with lesions covering 1/8 of the leaf area contained only 60% as much crude protein as disease-free leaves; leaves 1/4 to 1/2 diseased had 45% as much protein. Crude fiber content increased proportionately as crude protein decreased. These and other data reviewed by C. H. Hanson (Proc. Intern. Grassland Congr., 9th, 1965, in press) show that foliar diseases reduce quality and that resistance would usually improve quality by reducing leaf drop and increasing nutrient value of leaves still on the plant. Of course, disease resistance also improves stand establishment, yield, and persistence.

Coumestrol content. Recent studies indicated that foliar diseases were the principal causes for accumulation of the estrogen coumestrol in alfalfa (USDA Tech. Bull. 1333, 1965; Crop Sci. 4: 480-82, 1964). Insect infestation also caused some coumestrol accumulation. Coumestrol contents were low in the semiarid states California and Utah, which averaged 10.4 and 12.6 ppm., respectively, for 6 cuttings over a 2-year period. Highest values were obtained in Iowa, which averaged 125.4 ppm. Average values for other states were as follows: Kansas, 71.2 ppm.; Nebraska, 49.2 ppm.; Pennsylvania, 87.8; and

North Carolina, 52.0. Coumestrol contents in the respective states were roughly proportional to expected frequency of disease occurrence.

Differences in coumestrol content of alfalfa varieties were comparatively small but statistically significant (Table 1), and varieties were in approximate

Table 1.--Coumestrol content of 5 alfalfa varieties harvested at the 1/10-bloom stage, averaged over 3 cuttings in each year at 7 locations

<u>Varieties</u>	<u>Coumestrol (ppm.)</u>		
	<u>1960</u> %	<u>1961</u> %	<u>2-year average</u> %
Du Puits	52.9	48.4	50.7
Vernal	53.7	52.4	53.0
Buffalo	57.7	60.8	59.2
Ranger	62.9	58.0	60.4
Lahontan	71.9	65.4	68.7

order of susceptibility to foliar diseases. Lahontan had the highest coumestrol content; Du Puits and Vernal had the lowest. M. Stob, D. D. Stuthman, and E. M. Bickoff (Agron. Abstr., Oct.-Nov. 1965, p. 51) reported coumestrol concentrations of 20 to 30 ppm. in healthy alfalfa, but Loper and Hanson (Crop Sci. 4:480-82, 1964) reported less than 2 ppm. in healthy alfalfa forage produced in controlled environments. Infection with foliar diseases caused coumestrol to accumulate in the forage. Loper has since found more than 600 ppm. coumestrol in some disease-infected samples (unpublished).

From a practical viewpoint, one can anticipate increased coumestrol content of alfalfa forage when environment favors disease development. Also, a long interval between cuttings will favor buildup of disease and coumestrol. Green chop operations in which forage is fed directly to livestock are particularly subject to delays in forage removal if growth is dependent on rainfall. Unless surplus forage can be made into silage, hay, or haylage, increased production resulting from abundant rainfall will tend to delay harvest, thus increasing foliar disease incidence and coumestrol content. "Stockpiling" uncropped alfalfa forage, as is sometimes done in the fall for late pasturage, would also be expected to result in coumestrol accumulation if the environment favored disease buildup. Alfalfa that is poor in quality (low leaf percentage) because of disease infection is likely to be high in coumestrol and to have estrogenic properties. Likewise, good quality forage free of disease is likely to have little estrogenic activity. Discovery of the disease-coumestrol relationship will help stockmen obtain the most economical production from feeding alfalfa. Also, breeding disease and insect resistant alfalfas to obtain low coumestrol meal or hay for breeding animals appears to be practical if a low coumestrol product is needed.

Resistance to intensive management. C. C. Lowe, Cornell University (personal communication) is firmly convinced that "breeders have made and can make real contributions to improved alfalfa quality by selection of types that will survive and tolerate intensive management systems" without sacrificing yield. Such lines cut more frequently would be superior in quality because the forage is less mature. Some progress already has been made.

Saponins. In 1959, total saponin content was determined for 4 varieties of alfalfa--Buffalo, Ranger, Lahontan, and Vernal--in each of 3 cuttings at Ithaca, N.Y., University Park, Pa., Raleigh, N.C., Ames, Ia., Lincoln, Nebr., Manhattan, Kans. (one test each on dryland and irrigated farms), and Logan, Ut. (USDA, ARS-34-44, 1963). Lahontan was consistently lowest in saponin content (Table 2). A fifth variety, Du Puits, tested only at Logan, Ut., and in the two Kansas tests was higher in saponin than any of the varieties in the main test. Comparatively large differences among varieties suggested that total saponin content of alfalfa could be reduced by breeding.

Table 2.--Mean total saponin content of 4 alfalfa varieties at 8 locations averaged over 3 cuttings--expressed as percentage of dry matter

Variety	Kans. (dry- land)	Nebr.	N.C.	Pa.	Utah	Iowa	Kans. (irri- gated)	N.Y.	8 loca- tions
Buffalo	2.91	2.73	2.92	2.50	2.24	2.63	2.53	2.48	2.62
Lagontan	2.40	2.34	2.44	2.43	2.02	2.39	2.20	2.34	2.32
Ranger	2.76	2.78	2.64	2.56	2.28	2.49	2.47	2.61	2.57
Vernal	2.92	2.98	3.02	2.45	2.41	2.58	2.84	2.62	2.73

In a follow-up of the foregoing study, Pedersen et al. (to be presented at Internatl. Grasslands Conf., Helsinki, 1966) found that saponin fractions from Du Puits reduced the growth of the fungi Trichoderma sp. much more than did those from Lahontan (Table 3). Similar results were obtained with lettuce

Table 3.--Effect of 5 saponin fractions from Lahontan and Du Puits alfalfa separated on silica gel plates on growth of fungus species Trichoderma

Band	Diameter (mm)	
	Lahontan	Du Puits
1	54	30
2	26	10
3	49	13
4	58	20
5	58	59
Check	--	60

and chicks as testers. The difference in biological activity of saponins in the two varieties can presumably be caused by different amounts of each saponin or of different saponins. I will not attempt to cover the saponin work being done by Dr. Kohler's group at Albany, California.

It should be stated that saponins are probably not important in present-day chick rations because of the small amount of alfalfa meal used. Saponin content, however, appears to be genetically controlled and probably could be reduced by selection. A low saponin variety would probably increase the potential for alfalfa meal.

Protein and crude fiber contents. We compared the protein and crude fiber contents of 4 varieties--Buffalo, Vernal, Ranger, and Lahontan--from six states harvested three times at the 1/10-bloom stage (USDA Tech. Bul. 1333;

USDA, ARS-33-44). The average protein content of cuttings ranged from 14.9% for the first cutting of 1959 in Iowa to 28.2% for the second cutting of 1960 in North Carolina (Table 4). The average crude fiber content of cuttings ranged from 17.9% for the first cutting in 1960 in Utah to 38.3% for the first cutting in 1959 in Kansas (Table 5). Differences in protein content among varieties were very small but statistically significant (Table 6). Differences in crude fiber content among varieties were not significant (Table 7).

Table 4.--Protein content (%) of 4 alfalfa varieties (Buffalo, Ranger, Lahontan, Vernal) by cuttings at 6 locations in 1959 and 1960

Cutting	States						Locations combined
	Pa.	Nebr.	Utah	Iowa	N.C.	Kans.	
<u>1959</u>							
First	16.3	17.4	17.5	14.9	22.2	18.0	17.7
Second	19.4	21.0	17.7	16.6	23.3	17.3	19.2
Third	21.1	19.1	20.9	20.1	23.1	17.0	20.2
Average	18.9	19.2	18.7	17.2	22.9	17.4	19.1
<u>1960</u>							
First	21.5	17.2	18.8	18.5	22.4	19.4	19.6
Second	18.6	17.2	20.4	19.6	28.2	18.6	20.4
Third	18.3	18.3	17.5	22.6	24.5	19.5	20.1
Average	19.5	17.6	18.9	20.2	25.0	19.2	20.1

Table 5.--Crude fiber content (%) (same headings as in Table 4)

<u>1959</u>							
First	35.0	36.2	31.1	36.3	29.8	38.3	34.5
Second	29.7	30.1	29.8	28.9	26.1	33.4	29.7
Third	31.1	31.4	26.1	30.0	31.1	32.4	30.4
Average	31.9	32.6	29.0	31.7	29.0	34.7	31.5
<u>1960</u>							
First	30.0	31.9	17.9	31.2	22.4	26.0	26.6
Second	30.4	32.8	19.2	26.8	19.0	27.6	26.0
Third	25.7	32.0	23.7	26.6	23.4	28.4	26.6
Average	28.7	32.2	20.3	28.2	21.6	27.3	26.4

Table 6.--Protein content (%) of alfalfa variations for 3 cuttings, averaged over 6 locations and 2 years

Variety	Cuttings			Average
	First	Second	Third	
Buffalo	18.6	19.6	19.9	19.4
Ranger	18.8	20.1	20.3	19.7
Lahontan	18.8	19.4	19.7	19.3
Vernal	18.5	20.2	20.8	19.8
Average	18.7	19.8	20.2	19.6

We know that there is also plant-to-plant variation for protein content and that breeding alfalfa for higher protein and lower fiber contents is a realistic objective. However, for the varieties I have mentioned, factors

such as those associated with area of production, cuttings, years, and time of cutting affect protein and fiber contents much more than does the variety itself.

Table 7.--Crude fiber content (%) of alfalfa varieties at 1/10-bloom stage for 3 cuttings averaged over 6 locations and 2 years

Variety	Cuttings			Average
	First	Second	Third	
Buffalo	30.1	27.4	28.6	28.7
Ranger	30.4	27.8	28.5	28.9
Lahontan	30.5	28.0	29.6	29.4
Vernal	31.1	28.0	27.2	28.8
Average	30.5	27.8	28.5	29.0

Antimetabolites. According to F. C. Elliott (Quarterly Bul. Mich. Agr. Expt. Sta. 46(2):242-53, 1963) and Elliott and Olien (*ibid.* 46(4):507-11, 1964), plants contain both positive and negative components of nutrition for animals. If plants differ in these components and if part of the variation is heritable, then elimination of certain negative components by breeding might add greatly to nutritive value.

J. A. Schillinger (Doctoral Thesis, Mich. State Univ., 1965), working in Elliott's laboratory, studied a heritable antimetabolite found in a diploid line of Medicago falcata known to have low nutritive value. This water-soluble antimetabolite affected cellulolytic capacity of rumen microorganisms, retarded growth of weanling voles, and produced chlorotic symptoms in excised alfalfa shoots. The suppressive effect could be nullified by additions of glycine, aspartic acid, and glutamine in the in vitro dry matter digestibility tests. These amino acids were also successful antidotes in tests of vole growth response and alfalfa shoot bioassays. Partial recovery from the antimetabolic effect in dry-matter digestibility studies was obtained when coenzymes DPN and DPNH were used as antidotes.

It is significant that Elliott and coworkers have demonstrated heritable plant-to-plant differences for nutritive value in alfalfa which are genetically controlled.

Carotene content. C. T. Brackney (Masters' Thesis, Kansas State College, 1949) and others found heritable differences in alfalfa which appeared to be independent of resistance to potato leafhopper yellowing. It should be feasible to increase carotene content by direct breeding methods as well as by the indirect methods proposed earlier.

Digestibility. T. J. Reed and C. C. Lowe (Rept. 19th Alfalfa Improv. Conf., 1964) point out that in terms of ruminant-animal needs and in terms of the most abundant nutritive effect of forages, the energy aspects are by all odds the most significant in the animal-forage relation. To determine energy value, the following information is obtained: (1) digestibility, (2) proportion of absorbed energy that the animal puts into the products formed, and (3) dry-matter intake. By these standards, C. C. Lowe and coworkers at Cornell University have demonstrated differences among varieties (unpublished). H. G. Chaverra at Purdue University (Doctoral Thesis, 1965) studied general and specific combining-ability variances among 10 alfalfa clones for in vitro dry-

matter digestibility and several related characteristics and suggested a breeding program to develop higher digestibility in alfalfa.

Summary. We have noted that some breeding objectives, such as disease and insect resistance, indirectly improve quality of alfalfa forage. Research to modify the chemical composition of alfalfa by direct breeding methods is in its infancy.

Patterns of variation of four chemical constituents differed greatly in nationwide studies, and prospects of changing the content of each varied with constituent. Heritable differences in protein and fiber contents were small as compared to effects of environment and time of cutting. Total saponin content and kinds of saponin, on the other hand, appeared much more subject to direct genetic control and change through breeding. Coumestrol content was associated primarily with foliar disease infection. Growing alfalfa in regions with low frequency of disease or using a management system which keeps foliar disease to a minimum would be effective in producing low-coumestrol forage. Breeding for disease and insect resistance should receive first consideration if coumestrol contents are to be lowered by breeding. Heritable differences were noted for contents of an antimetabolite, carotene content, and digestibility.

ALFALFA CUTTING PRACTICES FOR QUALITY AND LONGEVITY

Dale Smith

Department of Agronomy, University of Wisconsin, Madison

Alfalfa is the most important forage used in the North Lake states. Minnesota, Wisconsin, Michigan, and New York harvested in 1964 about 8 million acres for hay, or 27% of the U.S. total. This acreage does not include the alfalfa used for pasture, for which accurate figures are not available. The growing season is short in these states and winters are severe. Management practices generally have been conservative in order to maintain persistent and high-yielding stands. As a result, forage quality has not always been the best. Greater emphasis needs to be placed on quality, since this is primarily a dairy area where high-quality forage is needed for high milk production.

A decade ago, most alfalfa varieties lacked high winter-hardiness and/or wilt resistance and conservative management was necessary to maintain persistent and productive stands. Alfalfa was allowed to go to full bloom and was cut twice, once in late June and again in late August. Delaying the first cutting to full bloom had several advantages: (1) plants were allowed time to heal winter injuries, (2) the incidence of bacterial wilt (*Corynebacterium insidiosum*) was reduced, (3) potato leafhopper (*Empoasca fabae*) invasion of the second crop was reduced, and (4) carbohydrate reserves in the roots were at a high level (Figure 1). These factors are still important today when varieties are used that lack hardiness and/or wilt resistance. Also cutting no later than September 1 is as important today as in the past, because it allows plants to have the autumn months to fully harden (Table 1). Cutting during the critical autumn period opens the stands to winterkilling because of lowered carbohydrate reserves in the roots and because little stubble is left to catch snow that is needed to insulate the plants from subfreezing air. Little alfalfa would be grown in the North Lake

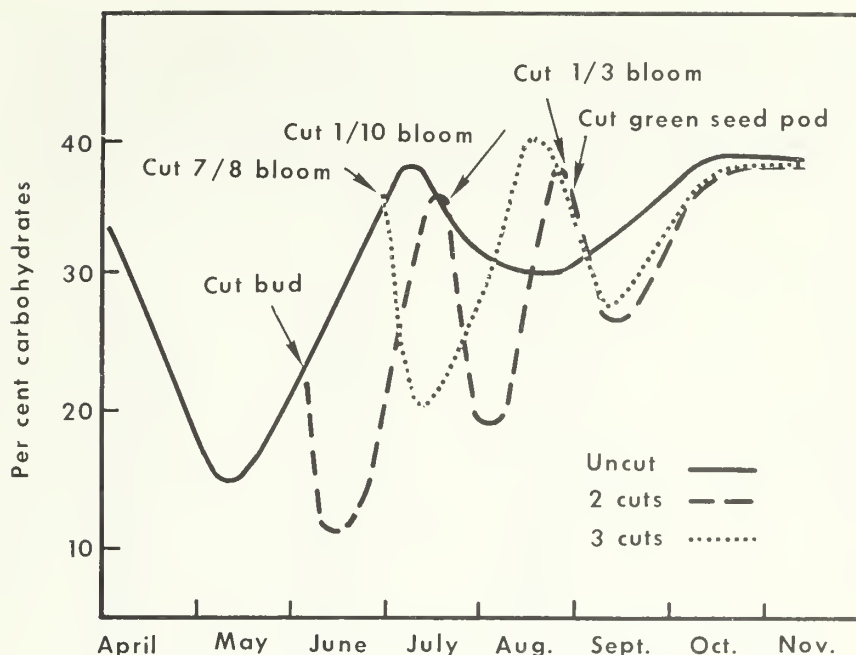


Figure 1. Seasonal trends of total available carbohydrates in the roots of Vernal alfalfa during 1960 at Madison, Wis., with two cuttings at mature stages, three cuttings at early maturity and with no cutting (Crop Sci. 2:75-78, 1962).

states without snow. Winterkilling usually occurs when snow is lacking, and this was particularly evident during the winter of 1964-65.

Table 1.--Comparative yields of alfalfa hay in the first harvest of 1960 following cutting at various dates during the previous autumn (Unpublished data, Wisconsin Agr. Expt. Station)

Fall treatment	Yields % of not cutting ^{1/}
Not cut	100
Cut Sept. 1	90
Cut Sept. 15	69
Cut Oct. 1	83
Cut Oct. 15	92
Cut Nov. 1	97

^{1/} Average over 10 varieties in 4 replications.

Cutting at full bloom, as practiced with the 2-cutting schedule, maintains stands in a productive condition, but hay quality is low as compared with cutting at earlier stages. Constituents important in animal nutrition, such as total digestible nutrients (TDN), sugars, protein, carotene, amino acids, and mineral elements, decrease in concentration with advance in maturity. Table 2 shows the composition of the spring growth of Vernal alfalfa harvested at prebud, midbud, 1/10 bloom, and full bloom. Animal feeding trials likewise

Table 2.--Chemical composition of the herbage of Vernal alfalfa at four stages of maturity of the spring growth (Wisconsin Agr. Expt. Sta. Res. Repts. 4,8,10, and 16, Crop Sci. 3:522, 1963, and Agr. Food Chem. 13:81, 1964, and doctoral dissertations. Similar data will be found in these publications for red and ladino clover, birdsfoot trefoil, and smooth brome grass.)^{1/}

Constituent	Prebud	Midbud	1/10 bloom	Full bloom
<u>Experiment A</u>				
TDN % ^{2/}	67.7	61.4	56.0	53.0
Nitrate (NO ₃) %	0.11	0.07	0.04	0.06
Crude protein %	26.5	23.3	17.9	15.8
Crude fiber %	21.0	27.9	34.0	35.4
Cellulose %	24.8	30.5	34.6	36.2
Ether extract (fat) %	1.8	1.5	1.5	1.4
Nitrogen free extract %	39.1	38.7	37.8	39.5
Total ash %	11.5	8.7	8.8	7.8
Phosphorus %	1.48	1.47	1.41	1.28
Potassium %	0.40	0.34	0.28	0.24
Iron ppm.	2.16	1.61	1.42	1.10
Manganese ppm.	310	184	154	153
Copper ppm.	58	50	52	46
Cobalt ppm.	7.8	6.8	5.8	6.0
Zinc ppm. ^{3/}	0.11	0.09	0.10	0.09
Carotene ppm. ^{4/}	31	29	23	24
pH of tissue ^{4/}	228	224	208	105
	5.58	5.46	5.65	5.50
<u>Experiment B</u>				
TAC % ^{5/}	15.7	13.6	11.1	13.3
Sucrose %	5.6	2.7	2.4	2.7
Glucose %	2.6	2.1	1.3	2.1
Fructose %	2.7	2.0	1.4	2.3
<u>Experiment C</u>				
Crude protein %	28.60	23.69	17.90	15.31
Alanine %	1.28	1.07	0.80	0.81
Arginine %	1.27	0.94	0.78	0.74
Aspartic acid %	3.28	- -	1.85	1.88
Glutamic acid %	2.20	2.07	1.42	1.53
Glycine %	1.14	0.94	0.76	0.77
Histidine %	0.55	0.42	0.37	0.35
Isoleucine %	1.07	0.88	0.69	0.68
Leucine %	1.82	1.53	1.20	1.17
Lysine %	1.41	1.10	0.90	0.87
Methionine %	0.40	0.30	0.21	0.22
Phenylalanine %	1.19	0.93	0.81	0.75
Proline %	1.00	0.89	0.75	0.69
Serine %	1.08	0.86	0.74	0.71
Threonine %	1.20	- -	0.78	0.61
Tyrosine %	0.82	0.62	0.54	0.51
Valine %	1.35	1.08	0.97	0.86

^{1/} Analyses based on two years on a dry matter basis from the spring growth of Vernal alfalfa at Madison, Wisconsin. Dry matter yields averaged over five years at these growth stages were 1898, 3304, 4648, and 5923 lb/acre and the average dates of these stages were May 16, May 28, June 10, and June 24.

^{2/} Total digestible nutrients from artificial rumen analyses.

^{3/} One year of data only.

^{4/} Analyses of fresh material.

^{5/} Total available carbohydrates (sugars, dextrans, starch) from takadiastase enzyme analyses.

have shown decreasing nutritive value of alfalfa as it matures. The higher gains of beef and sheep and the higher milk production from dairy cattle are shown in Table 3.

Table 3.--The value of alfalfa hay for meat and milk production as affected by stage of maturity

A. Yearling steers <u>1</u> /				
Items	Bud	1/10 bloom	Full bloom	Seed
Total gain per steer, lb. <u>1</u> /	96	69	58	44
Average daily gain, lb.	1.07	0.76	0.63	0.48
Feed for 100 lb. gain, lb.	959	1351	1600	2144
Adapted from Salmon, S. C., <u>et al.</u> , Kansas Agr. Expt. Sta. Tech. Bul. 15, 1925.				
<u>1</u> / 440 lb. initial weight.				
B. Lambs				
Items	Bud	Early bloom	Full bloom	Green seed
Dry matter digestibility, %	71.3	65.0	58.0	55.1
Daily gains, lb./acre	0.38	0.21	0.15	0.05
Adapted from Krueger, C. R., <u>et al.</u> , J. Dairy Sci. 46:631, 1963.				
C. Milk cattle				
Items	1/10 bud	Full bud	Full bloom	
Average eaten, lb./day	41.1	39.1	35.5	
Average 4% fat corrected milk, lb./day	29.8	27.0	24.7	
Adapted from Davis, R. F., and Decker, A. M., Grasslands, A.A.A.S., p. 225-230, 1959.				

Alfalfa harvested at an early stage of growth has a high content of nutritive constituents, but hay yields at immature stages are low. Thus, both composition and yields must be considered in a practical management system. The highest yield of most of the important chemical constituents is obtained at near the 1/10 bloom stage of growth. Although the yield of hay may continue to increase between 1/10 bloom and full bloom, it is due largely to an increase in the yield of fiber (Figure 2). The yield of protein and minerals may actually decrease after 1/10 bloom principally because of the loss of the lower, older leaves from diseases, lodging, and other factors. While the amounts of leaves (which contain a larger proportion of the nutrients than the stems) are decreasing, the upper internodes of the stems are continuing to elongate and the production of fiber continues to increase. Saving leaves has prime importance in hay making and this can be done best by cutting before many of the lower leaves are lost. The use of varieties resistant to leaf diseases is also important.

Harvesting at near 1/10 bloom is necessary to obtain high yields of nutrients per acre. This means that earlier cutting should be practiced in the North Lake states than the full bloom cutting that has been practiced in the past. Early cutting was not possible a decade or more ago because of the varieties being used, but it is now recommended because a high proportion of the alfalfa acreage is sown with the new, wilt-resistant and winterhardy

Erratum.--On page 12 of ARS 74-36, "Ninth Technical Alfalfa Conference Proceedings," a line of data for calcium is omitted from Table 2 and the data following are erroneous. Data for Experiment A are reproduced correctly below.

<u>Experiment A</u>				
TDN % <u>2/</u>	67.7	61.4	56.0	53.0
Nitrate (NO ₃) %	0.11	0.07	0.04	0.06
Crude protein %	26.5	23.3	17.9	15.8
Crude fiber %	21.0	27.9	34.0	35.4
Cellulose %	24.8	30.5	34.6	36.2
Ether extract (fat) %	1.8	1.5	1.5	1.4
Nitrogen free extract %	39.1	38.7	37.8	39.5
Total ash %	11.5	8.7	8.8	7.8
Calcium %	1.48	1.47	1.41	1.28
Phosphorus %	0.40	0.34	0.28	0.24
Potassium %	2.16	1.61	1.42	1.10
Iron ppm.	310	184	154	153
Manganese ppm.	58	50	52	46
Copper ppm.	7.8	6.8	5.8	6.0
Cobalt ppm.	0.11	0.09	0.10	0.09
Zinc ppm. <u>3/</u>	31	29	23	24
Carotene ppm. <u>4/</u>	228	224	208	105
pH of tissue <u>4/</u>	5.58	5.46	5.65	5.50

varieties, such as Vernal and Ranger. The hay-harvest schedule now recommended is 3 cuttings (June 1, July 15, August 30). The first cutting is made near June 1 at late-bud to 1/10 bloom, and this allows 2 more cuttings at an early bloom stage before September 1, the beginning of the critical autumn period.

The 3-cutting schedule provides the farmer with exceptionally high-quality forage, as well as high hay yields. Yields of protein and TDN per acre are higher than with 2 cuttings and as high as, or higher than, more frequent cuttings which often include harvesting during the critical autumn period. Yields from several management schedules are shown in Table 4. Yields of hay, protein, and TDN from 3 cuttings before September 1 were 20, 46, and 31% higher, respectively, than with 2 cuttings.

Table 4.--Yields from Vernal alfalfa harvested with various cutting schedules over a 3-year period, 1954-56, at Madison, Wis.
(Adapted from Wisconsin Agr. Expt. Sta. Res. Repts. 10, and 11, 1962)

Cutting schedules	Hay		Protein		TDN ^{2/}	
	Tons/ acre	% 2 cuts	Tons/ acre	% 2 cuts	Tons/ acre	% 2 cuts
2 cuts: Late June, late Aug.	4.44	100	0.75	100	2.47	100
3 cuts: Late April, late June, late Aug.	3.81	86	0.66	88	2.11	85
3 cuts: Late June, late Aug., early Oct.	4.70	106	0.86	114	2.67	108
3 cuts: Early June, mid-July, late Aug.	5.32	120	1.10	146	3.24	131
4 cuts: Early June, mid-July, late Aug., early Oct.	5.46	123	1.19	158	3.36	136

^{1/} Top dressed each year to maintain high soil fertility and insects controlled by spraying.

^{2/} Percent ETDN used from 1956 stage of growth studies.

Early and frequent cutting in the North Lake states should be practiced with certain precautions to maintain continuously productive stands. These precautions are listed below:

- (1) Cut only the winterhardy and bacterial wilt resistant varieties in this manner, e.g., Vernal, Ranger, 525, Progress, W.U. 202.
- (2) Fertilize adequately when the stand is established and topdress each year to at least replace the nutrients removed in the hay.
- (3) Spray the fields (usually the second crop) with an insecticide that is not harmful to livestock when insects are present and numerous enough.
- (4) When the stand has been winter injured, delay cutting the first crop until full bloom and harvest only two cuttings.
- (5) Remove the final cutting prior to the beginning of the critical fall period (usually September 1).

The 3-cutting schedule, however, has brought forth new problems. One is that harvests are based on calendar dates and disregard the fact that maturity on a particular date varies among varieties, years, and locations. The implementation of the 3-cutting schedule by date, however, aided the transition from

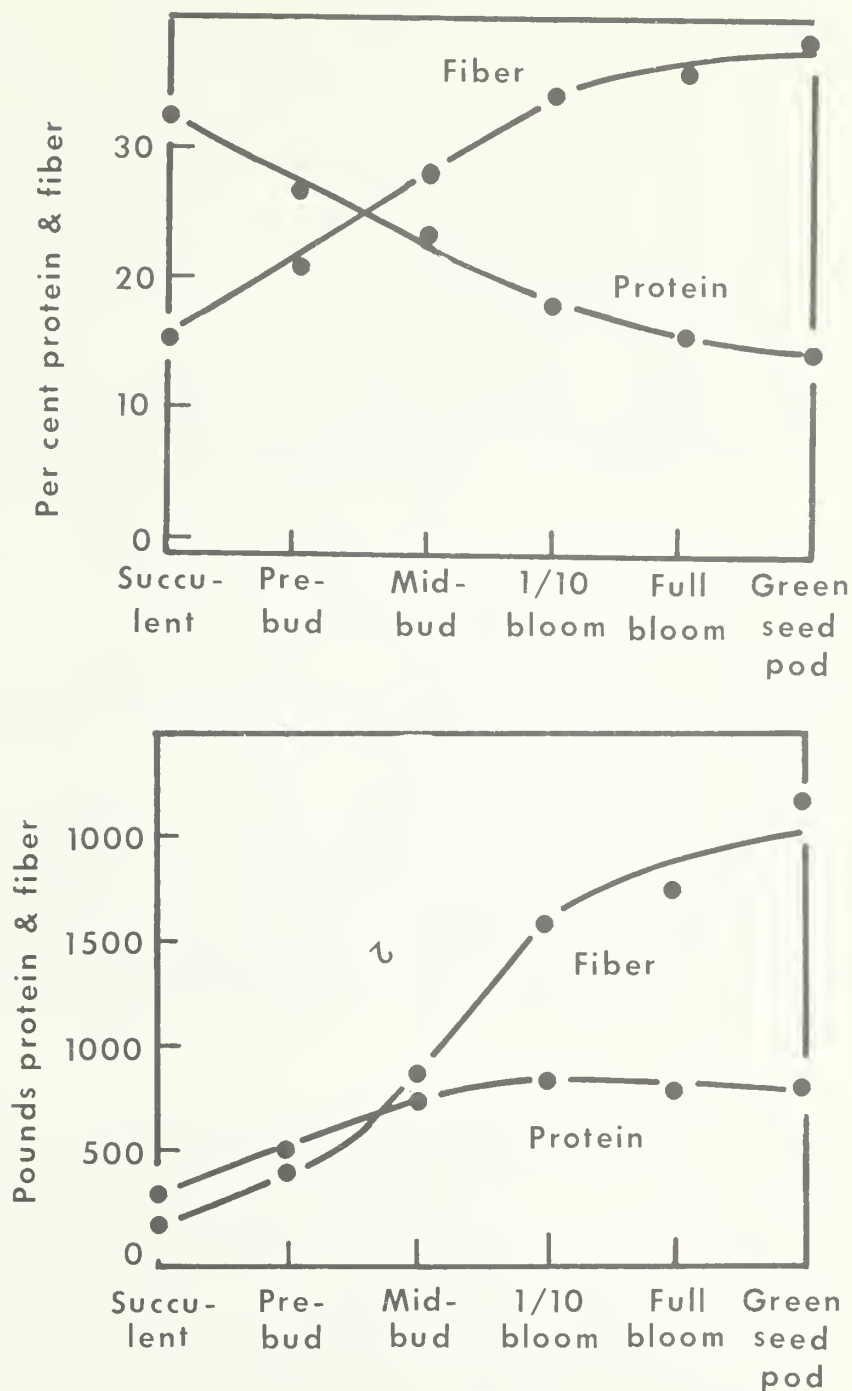


Figure 2. Percentages (top graph) and yields per acre (bottom graph) of crude protein and crude fiber in the spring growth of Vernal alfalfa at various stages of growth averaged for two years, 1955-56. Adapted from Wisconsin Agr. Expt. Sta. Res. Rept. 4, 1959.

the old 2-cutting schedule to one that emphasized early cutting to improve forage quality. It assured that the first cutting (June 1) was early enough to allow 3 harvests before September 1. Cutting on June 1 in southern Wisconsin closely approximates the appearance of the first flowers, and the remaining 2 harvests are also taken in early bloom. It is less satisfactory the further north in Wisconsin that it is used. This is primarily because the season is progressively shorter and the first cutting is harvested at progressively younger stages. Stands are weakened, and hay yields from 3 cuttings by date in northern Wisconsin (Ashland) are much reduced as compared with 2 cuttings (Table 5). The appearance of first flowers on the Vernal variety occurs about June 2 at Madison and June 4 at Arlington in the south but it does not occur until some 18 to 20 days later (about June 22) at Ashland in the north.

Table 5.--Yields in tons per acre from Vernal alfalfa cut 2 and 3 times averaged over 3 years at 3 locations south to north in Wisconsin (Wisconsin Agr. Expt. Sta. Res. Rept. 11, 1962)

Cutting schedule	Madison <u>1/</u>		Marshfield <u>1/</u>		Ashland <u>1/</u>	
	Hay	Protein	Hay	Protein	Hay	Protein
Late June and late Aug.	4.03	0.70	3.33	0.53	4.14	0.69
Early June, mid-July, and late Aug.	3.90	0.84	3.18	0.70	3.47	0.71
<u>1/</u> Marshfield is about 115 miles north of Madison. Ashland is about 140 miles north of Marshfield.						

A schedule based on plant maturity, instead of the calendar, would allow plants to indicate the proper time to cut, and would take into account variations in maturity due to varieties, years, and locations. It would allow the plants to determine how many cuttings are possible during a season. The appearance of the first flowers has been used in Wisconsin as the guide to stage of growth cutting. First flower closely approximates 1/10 bloom, and of equal importance, the first flower stage is much more easily recognized by the farmer than 1/10 bloom.

A management trial at 3 stations from north to south in Wisconsin has clearly indicated the practicability of cutting whenever first flowers occur (up to September 1) as compared with 3 cuttings by date (Table 6). Yields from the two schedules were similar in southern Wisconsin, but yields were progressively in favor of first flower cutting as the schedules were used further north. Thus, cutting at first flower has proved to be the most practical schedule over all conditions because it takes into consideration the progressively later maturity of the first crop from south to north. It also necessitates no line of demarcation below which 3 cuttings are suitable and above which they are not. The alfalfa itself is now used to indicate the proper time to cut. Over varieties and years, 3 to 4 cuttings at first flower are possible before September 1 in southern Wisconsin, while only 2 to 3 are possible in the north.

Another problem of the 3-cutting schedule (June 1, July 15, August 30) is that brome grass and timothy usually disappear from a mixture with alfalfa by the end of the first harvest season. These are grasses most generally sown with alfalfa in the North Lake states because they help to reduce soil erosion,

reduce winterkilling of the legume, and reduce the incidence of bloat of live-stock. The crux of the problem appears to be the June 1 cutting date. At this time, common brome grass is at about the boot stage and common timothy is jointing. Grasses are weakened by cutting at these stages when food reserves are at a low level. It also has been indicated that crown buds may not be developed enough to rapidly elongate and to produce a rapid recovery growth. Even so, the second and third growths of brome grass are usually sparse at best and consist of nonflowering tillers, while timothy may produce some flowering stems. As a result, the second and third growths of the rapidly growing alfalfa form a canopy over the grasses that excludes most of the light, and the alfalfa competes also for moisture and nutrients. The grasses thus have little chance of survival. Brome grass and timothy persist much better in mixture with alfalfa under the old 2-cutting system, where the grasses are close to maturity before cutting, and under frequent cutting or grazing, where the alfalfa stand is weakened and thinned. Only further research will answer the question of how to grow these grasses in the North Lake states in mixture with the new persistent alfalfa varieties and under management schedules that remove the first harvest at a vulnerable period for the grasses.

Table 6.--Yields in tons per acre from Vernal alfalfa cut by date
3 times and by first flower averaged over 2 years (1963-64)
at 3 locations south to north in Wisconsin
(Unpublished data, Wisconsin Agr. Expt. Station)

Cutting schedule 1/	Arlington 2/		Marshfield 2/		Ashland 2/	
	Hay	TDN	Hay	TDN	Hay	TDN
Date	4.69	2.85	3.63	2.40	2.44	1.57
Flower	4.31	2.71	4.09	2.68	3.02	1.70
LSD, 5%	0.15	- -	0.16	- -	0.11	- -

1/ Date schedule was June 1, July 15, August 30. Maturity schedule was cut whenever first flowers appeared to September 1 which gave 3 to 4 harvests at Arlington, 3 at Marshfield, and 2 to 3 harvests at Ashland.

2/ Marshfield is about 100 miles north of Arlington. Ashland is about 140 miles north of Marshfield.

SEPARATION -- NEBRASKA STAGE-OF-GROWTH STUDY

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and Economic Development)

There is a growing demand for a dehydrated alfalfa product of high nutritional value for monogastric animals. There is a special need for higher pigmentation potential in the dehydrated alfalfa which goes into broiler rations and layer feeds. There is need in these feeds for a product containing less fiber.

Use of American-made dehydrated alfalfa in foreign countries is expanding. To pay freight on tons of fiber, something the chickens and pigs can make no good use of, is economically as well as nutritionally unsound. This holds true

whether the travel is Cozad to Boston, San Francisco to Hong Kong, or Omaha to Caracas. We propose to show one method by which a much more satisfactory grade of dehydrated alfalfa can be made and to present some of the data accumulated this year in Nebraska with our mobile dehydrating and processing unit. Some of you are familiar with previous reports (Feedstuffs 36:60, 1964; Proc. 23rd Ann. Convention Amer. Dehy. Assoc., Jan. 1965).

We are aware that in making a superior grade for poultry and swine we will necessarily produce its complement, a product lower in protein and higher in fiber. We are confident that this lower-protein, higher-fiber product will be a better feed for cattle and sheep than most of the suncured hay or suncured meal now used. In pellet or cube form it will assure easy and economical handling, almost no loss in feeding, and a generally higher content of those factors which are lost in field curing and in field or bunk feeding. It has long been a mystery to me why 13% suncured alfalfa meal can be sold at times for as much as 5 or 6 dollars more than 17% dehy with 100,000 IUA guaranteed.

When the dehydration industry was young, dehydrators were constantly worried lest the development of a cheaper synthetic vitamin A would cause loss of market. This worry grew and as a consequence in the 1940's great activity was stimulated to protect carotene. This investigative activity led directly to cold storage, inert gas storage, and ethoxyquin, in that order. Without the two latter developments it is doubtful where the industry would be today, for they led directly to other improvements: pelleting, oiling or greasing, and bulk operations which resulted in economies of operation and greater acceptability of product.

New improved varieties of alfalfa, new and more effective pesticides, greater and more intelligent use of plant nutrients, and a better appreciation of control of the interval between cuttings have all contributed to a better green chop being delivered to the dehydrator. The average protein grade of dehydrated alfalfa in the past five years is probably 2 to 3% higher than it was in the years 1932-42.

No industry can rest on its laurels and continue in sound growth. That the alfalfa dehydrating industry recognizes this is evident in its continued support of a research program in its Association, a program now in its 17th year. Further evidence of their desire to keep abreast of new ideas and new accomplishments is the Association's joint sponsoring of these Technical Alfalfa Conferences since the very first one in 1951. I consider it my great good fortune to have attended and participated in all of the nine conferences.

In anticipation of the needs of the near future, the investigations reported herein were begun at USDA's utilization research laboratory at Albany in 1962, working with the Nebraska Department of Agriculture and Economic Development under a cooperative agreement. In that year and in 1963 and 1964 we had valuable assistance from several dehydrating firms in the Midwest and as far east as Pennsylvania. In 1964 and 1965 we worked closely with Dixon Dryer Company of California and V. C. Britton Company of the same state. This year under a cooperative agreement with Nebraska Farm Products Inc., of Cozad, Nebr., we for the first time took our experiments out of the laboratory and out of California to work directly with alfalfa growing in the state which leads in total production, Nebraska.

Our previous investigations of this nature have been performed on dehydrated chops shipped to us by cooperating dehydrators or picked up by ourselves at dehydrating plants and brought to Albany for processing. Separation was then made on these samples by feeding them to the disintegrator by hand and later feeding them to a screening device by hand. There are a number of objectionable features to such testing. First, the dried chops have either lost or gained moisture since they were collected at the plant. The leaf and stem become equilibrated in moisture content, thus losing some of the advantages of a differential. Second, it is virtually impossible to get a uniform flow to the screen by hand. Third, it is difficult to get a sample of chopped dried alfalfa at any mill which is as truly representative of the entire run as is desired. Any study of the carotenoid pigments is of less value.

Last year we acquired a small triple pass dehydrator with a capacity of about one-fifteenth to one-tenth that of the average commercial machine. We also acquired a 40-foot flat-bed trailer from Navy surplus. Our engineer, Alex Mottola, mounted the dehydrator, disintegrator, and auxiliary equipment on this trailer, installed thermometers and thermocouples, and provided partial automatic controls. The outfit was given trial runs at the laboratory and later at the Dixon Dryer Company's plant about 50 miles east of Albany. It was returned to Albany in the fall and modifications were made to make it suitable to move to Nebraska in June. Everything connected with the processing can be loaded on the trailer with the exception of the automatic feeder(see Fig. 1).

In Cozad our gas supply was at 20 pounds ahead of our own regulator. Previous trials had all been done with gas pressure at a maximum of 6 pounds. Our maximum production rate this summer was made on a 9-hour run and was 261 pounds commercial dry weight per hour or 1,084 pounds of water evaporated per hour. On short runs of plot material, rate was reduced to about 130 pounds per hour, dry weight.

In our work at Cozad we operated on second and third cuttings of a field of Ranger variety seeded in 1963. The field is fairly well drained, level and uniform in growth appearance. It lies just across a farm lane from some University of Nebraska variety plots and is a part of the Knoedler farm. The first cutting had been removed on May 17. We laid out a plot 56 x 441 feet in the center of the field. Nebraska Farm Products, Inc., our cooperators, did all of the harvesting though they did at times call upon equipment from Lemmon Mills at Willow Island.

The plan of the experiment was to cut two 7-foot swaths, one on each side of the main plot on each cutting date. Cuttings were 4 days apart in the first part of the experiment but lengthened to 8 days in the second part, though the age of growth was 28, 32, 36, and 40 days, respectively, on both second and third harvest. On two occasions weather prevented our cutting on the exact date predetermined. Plot 1 was harvested one day late on third harvest and plot 2 was cut one day early.

The four cuttings made in the first part of the experiment were very exact and upon finishing plot 4 we found only a very narrow strip of alfalfa remaining, not more than 6 to 10 square feet at most. During the second part, while on third harvest material, we experienced a great deal of rainfall. Cutting a truly straight swath when the ground surface is very wet is difficult.

We have found it necessary to make some adjustments in calculation of area harvested on plots 2, 3, and 4. In the first half each cutting of each plot is calculated as 14.17% of one acre. In the second half the plots are calculated as 14.17, 13.67, 13.41, and 15.44%, our best estimate of actual areas cut and processed.

The alfalfa was harvested with a Fox field chopping unit, tractor drawn, blowing directly into a covered trailer. The loads of green chop were weighed at the plant and the empty trailer weighed at the end of the run to obtain net wet weight processed. Our dehydrator is equipped with a feeder hand controlled for rate of feed to the dehydrator. The green chop was fed by hand into the automatic feeder.

The semiautomatic controls on the dehydrator allowed us to operate at a fixed outlet temperature but maintained no control on inlet temperature other than hand regulation of feed rate to maintain the "feed more, feed less" needle in a nearly constant position. We maintained an outlet temperature of 250°F. Feeding to control inlet temperature and rate of production is an art and requires careful attention to the state of the green chop in the feeder to provide constant level and as nearly as possible constant compaction before the material is carried forward to the leveling reel and the kick-off reel.

After the alfalfa is dehydrated it passes through the outlet fan to the cyclone separator for release of the hot moisture-laden air and the dry chops are dropped into a small screw feeder which delivers to the disintegrator. This disintegrator is a vertical hammer mill with rigid hammers. No screen was used as we wanted only to shatter leaves and break leaves loose from stem, with the least possible fracturing of the stem itself. An important consideration here is to dehydrate in such a manner that the stem material leaves the dehydrator with a fairly high moisture content while the leaf material is practically bone dry. In our work this summer we are certain that this goal was not as well accomplished as it might have been if further improvements in controls had been made.

The dried chops fall directly from the disintegrator onto a shaking device or scalper. In our set-up, where the disintegrator is mounted on the trailer but the screening device stands on the ground level, it was necessary to provide a solid sheet-metal extension to our screen. This undoubtedly changed the vibration characteristics of our scalper but it seemed to do a good job. The shaker itself is equipped with two feet of 100-mesh woven-wire cloth at the upper end followed by 6 feet of punched-steel screen with round perforations of 6/64-inch diameter. The material passing through the 100-mesh screen contains most of the grit or soil and in our Nebraska work averaged about 1.2% of the whole. In all cases we have included it in the leaf fraction in our calculations. Some samples in previous experiments have been so high in grit and the grit so high in ash that we have felt it would be advantageous to discard it as means of protecting processing machinery. We still believe this would hold true in some areas of production. The leaf material falls through the 6/64 openings and the stem slides off the end of the scalper. All fractions were collected in bags and weighed at the end of each run.

Generous samples of each fraction were taken. These were cut down on a riffle, ground to pass 40 mesh, and sent to our laboratory for complete feeds analysis and carotene and xanthophyll. At the time cuttings were made, samples of the growing plant were taken from the field. A careful, and I may say tedious, hand separation was made of leaf and stem. These samples were dried in a vacuum oven, prepared for analysis, and sent to our laboratory for complete feeds analysis but not for carotenoid pigments.

In this report we have converted all analytical results to a 93% solids basis as being more realistic to the owners and operators of dehydration equipment for alfalfa. The project is divided into two phases. One objective was to determine as well as we could the stage of growth of Ranger variety under local conditions for maximum utilization of the growing crop when it is dehydrated. The other objective was to separate the dehydrated crop into two fractions, one for monogastric and one for ruminant animals. The two objectives are somewhat interdependent.

Table 1.--Increase in production of alfalfa and of protein

	Pounds alfalfa	% Increase over previous plot	Pounds protein	% Increase over previous plot
Plot 1, 28 days	4147	--	841	--
2, 32 "	4897	17	917	9
3, 26 "	5475	12	1012	10
4, 40 "	6059	11	1045	3
Cumulative increase	1885	45	204	24

Another way to observe this is in Figure 1 which shows not only the alfalfa and protein but also fiber, carotene and xanthophyll.

Table 2.--Increases for carotene and xanthophyll

	Grams carotene	% Increase over previous plot	Grams xanthophyll	% Increase over previous plot
Plot 1, 28 days	422		507	
2, 32 "	440	4	546	8
3, 36 "	444	1	552	1
4, 40 "	480	8	595	8
Cumulative increase	58	14	88	17

Table 3.--Increases for fiber

	Pounds fiber	% Increase over previous plot
Plot 1, 28 days	1133	
2, 32 "	1388	23
3, 36 "	1629	17
4, 40 "	1905	17
Cumulative increase	772	68

It might be concluded from these figures that a cutting cycle of somewhere near 36 to 40 days would be the best practice on second and third cuttings of

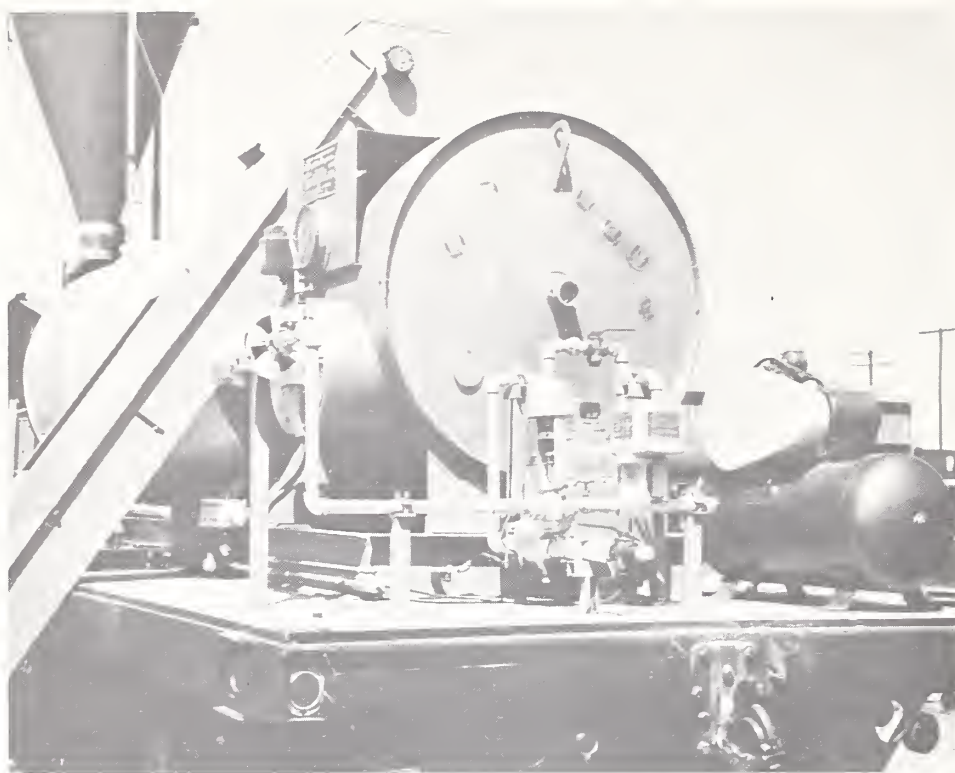


Figure 1. Firing end of mobile dehydrator.

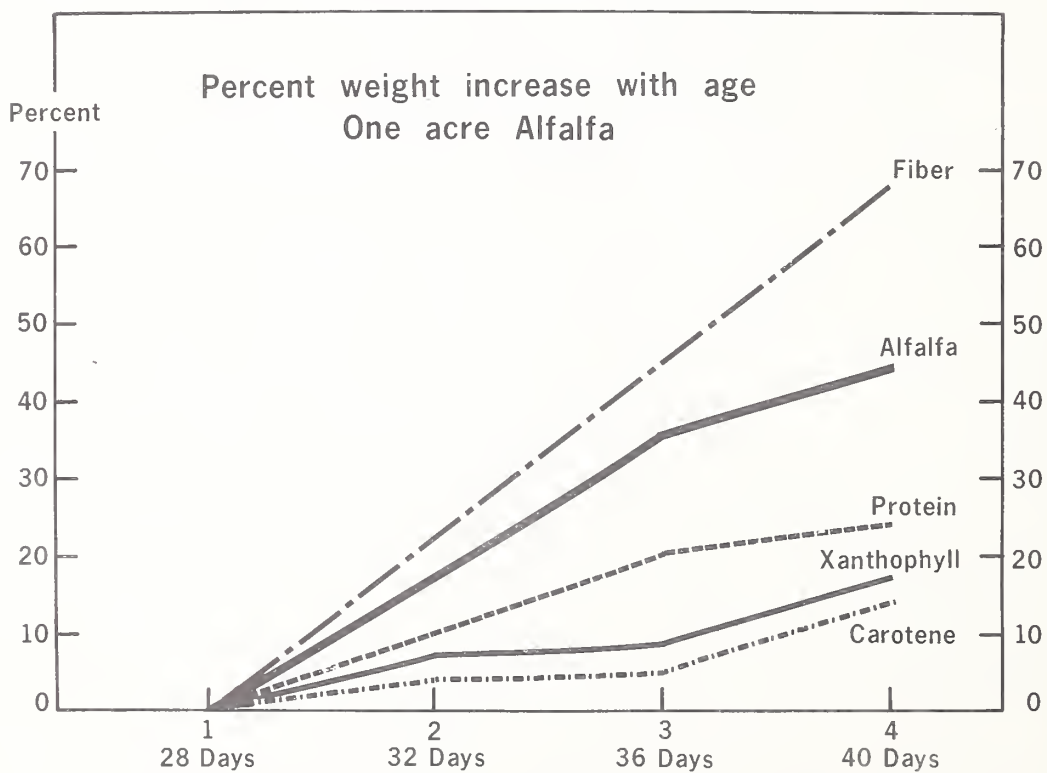


Figure 2. Weight increase with age - one acre of alfalfa.

Ranger alfalfa in the area of Cozad, Nebr. Total tonnage, protein, carotene, and xanthophyll all increased in total weight per acre between the 36- and 40-day plot although protein increase did not parallel that of tonnage. Nothing seems to proliferate like crude fiber. It appears to increase in total weight about 1.7% per day between the 28-day and the 40-day plot in this experiment.

The quality of the whole alfalfa and of each fraction is of great interest in this attempt to get a high-protein, low-fiber fraction for poultry and swine. Tables 4 to 6 show the percentages of the whole by weight and their protein, fiber, carotene, and xanthophyll content.

Table 4.--Whole alfalfa

	Weight %	Protein %	Fiber %	Carotene mg/lb	Xanthophyll mg/lb
Plot 1, 28 days	100	20.1	27.1	101	121
2, 32 "	100	18.7	28.3	90	111
3, 36 "	100	18.5	29.8	81	101
4, 40 "	100	17.2	31.4	79	98
Weighted average	100	18.5	29.4	87	107

Table 5.--Leaf fraction

	Weight %	Protein %	Fiber %	Carotene mg/lb	Xanthophyll mg/lb
Plot 1, 28 days	57	26.6	16.7	158	176
2, 32 "	50	25.1	17.9	137	158
3, 36 "	50	25.2	17.5	128	150
4, 40 "	48	24.5	18.0	131	148
Weighted average	50	25.3	17.6	138	157

Table 6.--Stem fraction

	Weight %	Protein %	Fiber %	Carotene mg/lb	Xanthophyll mg/lb
Plot 1, 28 days	43	13.8	37.7	47	68
2, 32 "	50	12.5	38.6	43	65
3, 36 "	50	11.8	41.8	35	52
4, 40 "	52	10.6	43.6	32	53
Weighted average	50	12.0	40.9	38	58

To summarize these tables we may say that from alfalfa of 18.5% protein we made about half into a 25% protein grade and left about half as a 12%. By using screen openings of less than 5/64-inch one could reduce the weight of the leaf fraction to perhaps 25% of the whole and improve the protein content of each fraction by leaving more of the stem protein with the stem fraction and also leaving more of its fiber there. Until we are able to evaluate grades of alfalfa on their true values in feeding, including metabolizable energy and pigmenting ability, it will be impossible to state conclusively the worth of each grade in dollars.

Another possibility is that of increasing the screen opening to scalp off a very small portion of stem in order to raise the grade of the remaining product a very few percentage points. We were limited in the scope of this project by having a fixed screen-size opening.

Some of the byproducts of our summer's work in Cozad were the production of two tons of stem fraction of Ranger alfalfa harvested on an adjoining field of much the same characteristics as our plot field. These two tons as well as about a ton of leaf fraction have been delivered to the Department of Animal Science at the University of Nebraska for use in their studies on digestibility of fiber. We also processed lesser quantities of three experimental varieties, two of them of Flemish type and one of Lahontan and one of Gold and Pure. Portions of some of these are being used in the Western laboratory in saponin studies.

In an attempt to evaluate the efficiency of the process we have applied a formula used for many years for determining Ratio of Concentration and Apparent Recovery of the valuable metallic content of an ore upon milling. This is a rapid way of determining what is being accomplished before the weights of the fractions are known. A study of actual weights and analyses has proved the formula to be very dependable. Table 7 illustrates the ratio of concentration and apparent recovery of protein by both hand and mechanical separation.

Table 7.--Concentration and recovery of protein

	Hand		Mechanical	
	Ratio	Recovery, %	Ratio	Recovery, %
Plot 1, 28 days	2.54	61.3	1.76	71.9
2, 32 "	2.70	61.7	2.01	66.4
3, 36 "	2.65	62.9	2.02	69.1
4, 40 "	2.81	61.2	2.10	67.7
Average	2.68	61.8	1.98	68.7

You will note that in hand-separated samples the ratio of concentration is higher and the recovery lower than in mechanical separation. In the mechanical separation some of the stem protein is in the leaf fraction, so that its protein concentration is lowered and the total recovery of protein is higher. The same formula can be applied to other factors which we wish to concentrate in the leaf fraction.

On the first four plots and during erection of the machine in Nebraska, Ken Smith of the Western laboratory assisted. Alan Zwink's services were supplied by the Nebraska Department of Agriculture. Management and employees of Nebraska Farm Products, Inc., Kansas-Nebraska Natural Gas Company, Cozad Light and Water Company helped us. During the long run in the second division of the project, John W. Nelson took charge of the operation in a creditable manner. As supervisor I was there throughout the experiment and handled all sampling and sample preparation. Richard Knowles of the Western laboratory did all of the carotenoid analyses. Archie Curtice and Elton Griffis of Cozad dug a well for us and installed a pump to supply water for our vacuum oven. Darrold Termunde of Pfister Associated Growers, Inc., provided cuttings from two Flemish type varieties.

COMPOSITION OF STANDARD AND SUPER GRADES OF ALFALFA

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During the past year we have been engaged in an exhaustive study of alfalfa composition with particular reference to amino acid analyses and to structural components which might be used to estimate metabolizable energy. This work was partially supported by the American Dehydrators Association whose grant permitted hiring a summer employee for this work. The ADA also supplied us with aliquots of the same samples of market-grade blends used in the Midwest Research Institute assay project reported on by the ADA earlier this year. These samples ranged from 15 to 22% protein. To these we added four samples including three "super" grades running about 25, 27.5, and 30% protein (dry basis) and the sample of 22% protein meal which was the raw material for the three super-grade samples. The 30% protein material was the leaf fraction made as described by Mr. Chrisman. The 25 and 27% protein samples were blends of leaf fraction with whole unfractionated meal.

Nutritionists have long recognized that the old crude-fiber method was essentially useless as a direct measure of indigestible material in feeds. It is well known that the crude fiber analytical procedure results in destruction of substantial amounts of lignin, cellulose, and pentosans and thus does not include all structural constituents of the plant which are not usable by the chick, for example, as an energy source. If crude fiber and ash were the only non-energy-producing components of dehydrated alfalfa, a 17% protein grade should have a metabolizable energy value for the chick of close to 1000 calories instead of slightly more than half that figure as determined by biological assay.

We have been working on a nondestructive procedure for estimating these components which could be used to correlate with direct measurements of metabolizable or productive energy for various types of livestock. Metabolizable energy values are being determined on this series of samples by Dr. Summers and coworkers at Ontario Agricultural College, and digestibility studies on four of the samples are being carried out on sheep by Dr. Ammerman and colleagues at Florida Agricultural Experiment Station. I should like to show you one of the chemical approaches we have been taking and present results on the series of samples I have described. Then I will present some results on amino acid analyses by procedure we have been developing.

The analytical scheme in Figure 1 has been adapted from earlier work on hays. The fatty material and some water-soluble materials are extracted first with benzene-ethanol solution and this solution is discarded. The residue is extracted with a dilute solution of ammonium oxalate in water, which removes all of the water-soluble materials including pectin in the form of ammonium pectate. At this stage the residue contains all of the cellulose and lignin as well as the insoluble fraction of the pentosans, proteins, and ash. Published methods usually follow with a series of sodium chlorite treatments, designed to break up and solubilize the lignin and to leave the cellulose intact. With alfalfa samples we have found that this step does not completely remove lignin and does destroy substantial amounts of pentosans and celluloses. We have therefore omitted it from our procedure and resorted to analysis of the oxalate

residue for residual protein, pentosans, lignin, ash, and fat. Thus we arrive at a figure we define as WRRL cellulose.

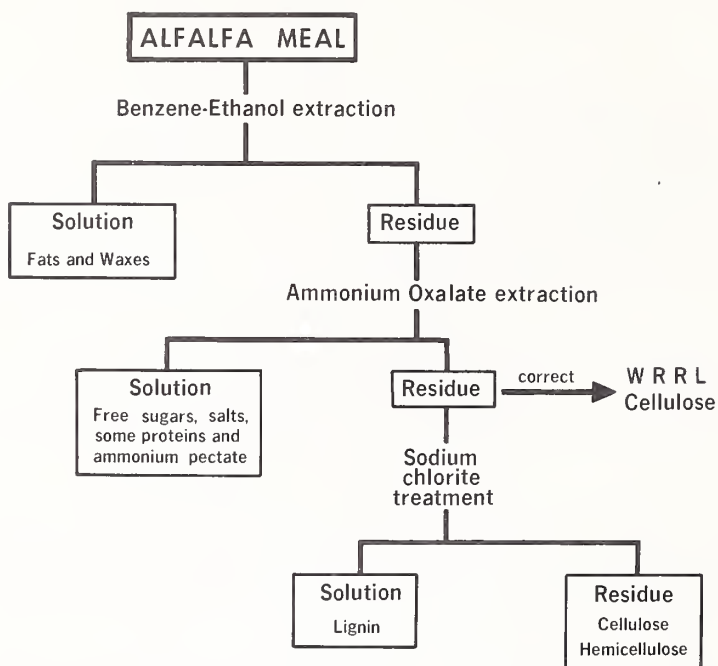


Figure 1. Analytical scheme.

Figure 2 shows the results of applying this procedure to the dehydrated alfalfas of different protein contents. It will be noted that WRRL cellulose dropped from over 25% in the 15% protein grade sample (16.5% protein on a dry basis) to about 12% in the highest super-grade sample (30.4% protein on a dry basis). The figure also shows that lignin and pentosans decreased as protein content increased and that ash was relatively constant in the various grades. Since the components shown in Figure 1 (e.g., WRRL cellulose, pentosans, lignin, and ash) represent distinct nonoverlapping fractions, they can be added together for each sample and the sum should represent the relatively indigestible material of alfalfa for chicks (e.g., WRRL nonnutrients shown in Figure 3). This sum subtracted from 100, of course, should represent the digestible components which are plotted in Figure 3 as "WRRL nutrients." Crude fiber data are included for comparison. It will be noted that the WRRL nutrients range from about 37% in the lowest-protein sample to about 59% in the highest-protein super-grade sample. Crude fiber figures range from about 40% of WRRL non-nutrients in the highest-grade sample to 53% in the lowest-grade sample. One obvious unsolved problem in this procedure is that the pectin of the alfalfa is included in the WRRL nutrients. The error is not large since the pectin content of alfalfa is small. However, work is in progress to eliminate it from this fraction, since chicks apparently are unable to utilize pectin effectively as an energy source. It appears that the procedure does give results which fall in the range expected if the digestible-nutrient fraction of alfalfa produces energy comparable to corn (e.g., 1,560 cal/g.). Correlations will have to await completion of the metabolizable energy studies on the same samples. Other combinations of fractions may be used to correlate with digestibility by ruminants.

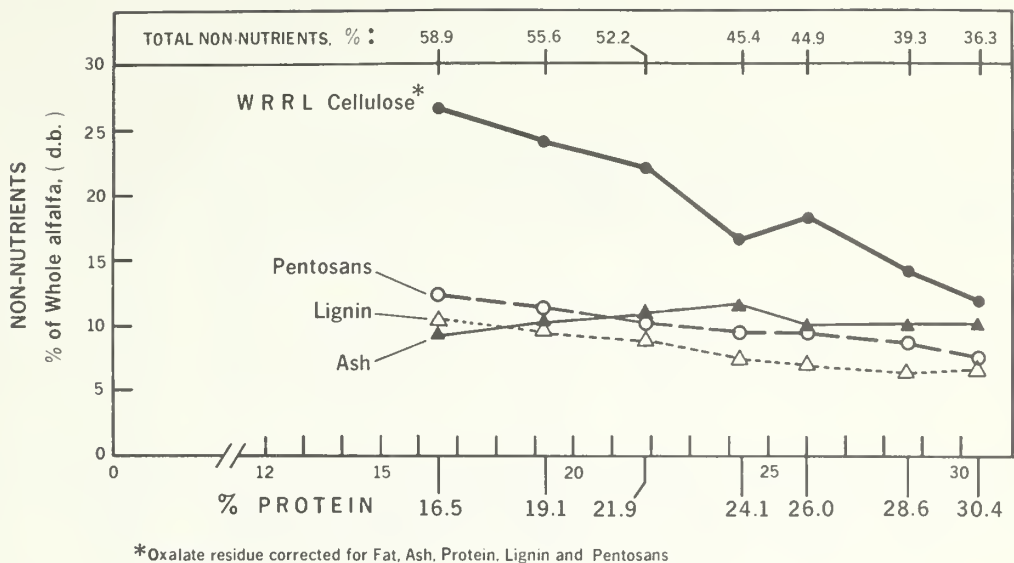


Figure 2. Nonnutrients, percent of whole alfalfa (d.b.).

Let me turn now to amino acid composition of alfalfa. There has been a great deal of research on methods for determining amino acids and it is generally conceded that each type of material presents its own set of problems particularly in method of hydrolysis. We have carefully studied hydrolysis procedures for alfalfa and developed a series of correction factors for amino acids which are partly lost during hydrolysis (e.g., serine, threonine, tyrosine) and for those which are slowly released during hydrolysis (e.g., isoleucine and valine). Cystine and methionine, which are partially destroyed by

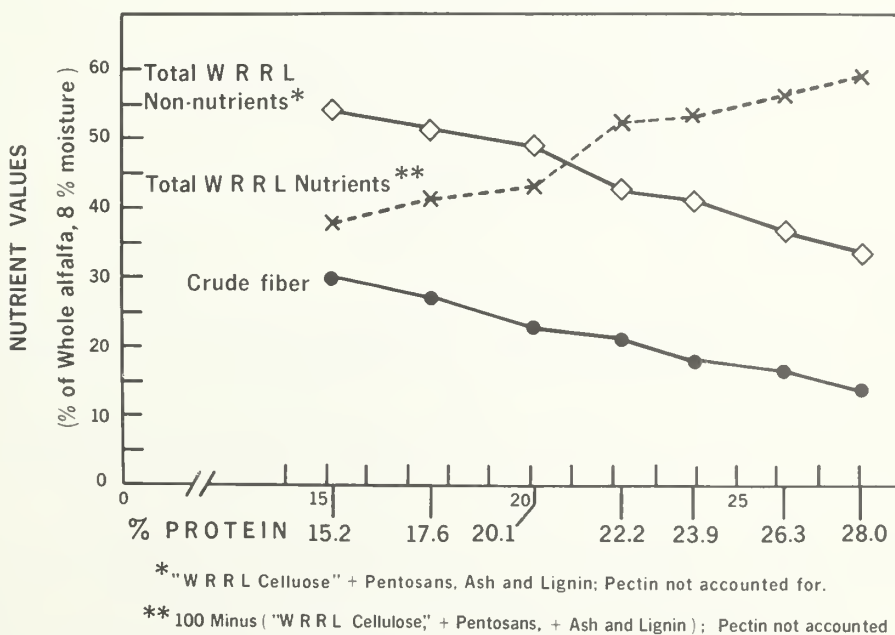


Figure 3. Comparison of nutrient values as percent of whole alfalfa, 8% moisture.

oxidation during the usual acid hydrolysis, were determined by the performic acid oxidation procedure of S. Moore (J. Biol. Chem. 238, 235, 1963). The essential amino acid results are shown in Table 1. The results indicate that,

Table 1.--Essential amino acids of different grades of dehydrated alfalfa meal (g./16 g. N)

Sample source Protein grade:	ADA				WRRL		
	15%	17%	20%	22%	25%	27%	30%
Lysine	3.9	4.0	4.2	4.6	4.8	4.5	4.5
Histidine	2.0	1.9	2.0	2.1	2.2	2.0	2.2
Arginine	3.8	4.1	4.7	4.5	4.9	4.6	5.0
Threonine	3.9	4.2	4.3	4.7	4.6	4.5	4.6
Cystine	1.1	1.0	1.1	1.1	1.1	1.0	1.0
Methionine	1.5	1.6	1.6	1.7	1.8	1.7	1.8
Glycine	4.7	4.9	4.9	5.4	5.1	5.1	5.2
Valine	5.5	5.8	5.8	6.3	6.2	6.1	6.1
Isoleucine	4.5	4.6	4.8	5.1	5.0	5.0	5.1
Leucine	6.9	7.3	7.5	8.2	8.1	8.1	8.1
Tyrosine	2.7	3.0	3.1	3.1	3.1	3.0	3.3
Phenylalanine	4.3	5.0	5.1	5.6	5.3	5.5	5.2

with few exceptions, as protein content increases there is an increase in concentration of amino acid in the protein. That is, protein quality improves as protein content increases.

In conclusion, both carbohydrate and amino acid studies indicate that as protein content of alfalfa products increases, product nutritional value increases at a greater rate. Hence the dollar value of the super grades should be greater than estimated on the basis of protein or crude-fiber content.

UTILIZATION OF ALFALFA STEM CARBOHYDRATES

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The recent interest of alfalfa dehydrators in the separation of alfalfa leaves and stems following the dehydration process has presented a new challenge. The leaves, which will contain 26 to 30% protein, will have a ready acceptance by the poultry and swine industries, not to mention others. The stems, which will represent approximately half (depending upon maturity) of the total material, do not have a demanding market. Thus, it became a challenge to investigate ways of processing the alfalfa stem fraction to improve its nutritive value.

The problem. Approximately 70% of the alfalfa stem fraction is carbohydrates. Another 10 to 12% is lignin, with protein, ash, and fat making up the remainder. The carbohydrate fraction approximates 10% soluble sugars, 50% cellulose, and 40% hemicellulose. All of these components are highly digested by rumen microflora when in their pure state. However, when associated with lignin in the plant, cellulose and hemicellulose digestibility is lowered.

Evidently cellulose and hemicellulose (holocellulose) respond similarly in this situation. It is quite well established that lignin inhibits cellulose digestion by an encrusting action based upon the deposition of lignin around the cellulose during the growth of the plant. The extent of this encrusting action is dependent upon stage of maturity. The more mature the plant, the greater the lignin content and the lower the cellulose digestibility. This is directly associated with a higher ratio of stems to leaves in alfalfa as the plant matures. The lignin and cellulose are structural components of the plant and thus occur most abundantly in the stem fraction.

Work of Terry and Tilley (*J. Brit. Grasslands Soc.* 19:363-372, 1964) show the effect of maturity on leaf-to-stem ratio and dry matter digestibility of the parts (Table 1). The digestibility of the leaves did not change, whereas

Table 1.--Dry matter digestibility (*in vitro*) and nitrogen content of leaf and stem fractions at different stages of growth (Terry and Tilley, 1964)

Date of cutting	Leaf %	Digestibility, %			Nitrogen content, %		
		Whole	Leaf	Stem	Whole	Leaf	Stem
April 2	70	84	83	85	4.8	5.3	3.9
April 16	57	83	83	82	4.3	5.0	3.2
May 1	50	79	83	76	3.5	4.6	2.5
May 14	40	72	82	68	3.1	4.6	2.1
June 4	36	68	81	59	2.5	4.1	1.6
June 25	35	63	82	56	2.3	3.8	1.6

the digestibility of the stems decreased with advance in maturity. Kamstra, Moxon, and Bentley (*J. Animal Sci.* 17:199-208, 1958) compared cellulose digestibility in alfalfa leaves and stems and associated each with lignin content (Table 2). They isolated cellulose from the plant parts and found an increase

Table 2.--Digestion of cellulose (*in vitro*) in leaf and stem fractions (Kamstra *et al.*, 1958)

Plant part	Acid lignin %	<i>In vitro</i> digestibility		
		Cellulose (whole plant)	Cellulose (isolated)	Holocellulose
Leaves	5.7	67.8	83.4	79.6
Stems	18.5	44.4	80.2	71.5

in digestibility when removed from the presence of lignin. The improvement in digestibility was directly proportional to the lignin content.

The research. The Nebraska Station with the cooperation of the Western Regional Research Laboratory, USDA, Albany, Calif., recently initiated a three-year study in which physical, chemical, and biological treatments will be tested to determine their ability to degrade lignin in alfalfa stem forage. *In vitro* cellulose and dry matter digestibility will be used as a measure for improvement.

Physical treatments. Some of the physical treatments considered for study are ball milling, autoclaving, heating, freezing, sonic vibrating, blending, and pelleting. Dehority and Johnson (*J. Dairy Sci.* 44:2242-49, 1961) studied the

effect of ball milling on cellulose digestion in several forages. Alfalfa, ball milled for 72 hours, has greater in vitro cellulose digestion than alfalfa ground through a 40-mesh screen (Figure 1). Ball milling was one of the first treatments used in the Nebraska study. The results are in agreement with those of Dehority and Johnson (Figure 2) except that a greater increase in digestibility was noted. The explanation would be that Dehority and Johnson used the whole plant, whereas alfalfa stems were used in the later study. Thus, there was more room for improvement. These two sets of data support the theory that a physical barrier exists between the cellulose and the cellulolytic bacteria in the rumen. Ball milling is a physical treatment and does not change the chemical structure of any of the constituents in the forage. Therefore, the lignin and other chemical components are present in the fermentation mixture and only the physical relationship has been altered.

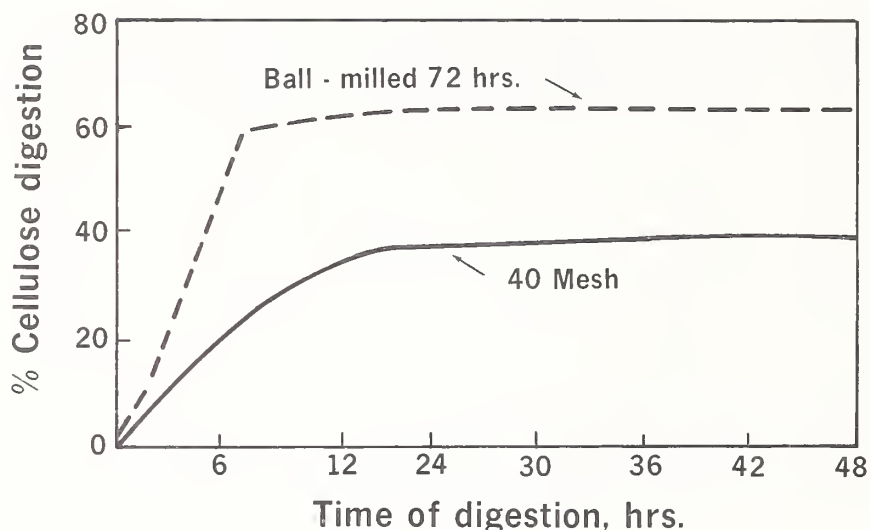


Figure 1. Effect of ball milling on cellulose digestion.

Chemical treatments. Sodium hydroxide, ammonium hydroxide, chlorine dioxide, strong acids, sulfite, ethanolamine and many other chemicals used in the paper industry have lignin-degradation properties which may have application to this problem. Beckmann (Chem. Abstr. 16:765, 1922) used alkali treatments to improve the nutritive value of wheat straw. One-hundred units of chopped straw treated for at least 4 hours with 8-fold its weight of 1.5% aqueous solution of sodium hydroxide at atmospheric temperature and pressure yielded 75 units of material with a nutritive value equal to 56 units of starch.

More recent work of Wilson and Pigden (Can. J. Animal Sci. 44:122, 1964) showed essentially the same results (Figure 3). They attempted to simplify the procedure of Beckmann by using a dry process and eliminated the final washing step to remove residual sodium hydroxide from the material.

Sullivan and Hershberger (Science 130:1252, 1959) studied the effect of chlorine dioxide on lignin content and cellulose digestibility of forages. In their laboratory experiments, air was bubbled through a solution of sodium chlorite, sodium acetate, and acetic anhydride and then passed through a glass tube containing 5 to 10 g. of finely ground roughage in an air-dry state for 4 to

24 hours. The acid odor of the air-dry sample could be removed by exposure to air, by vacuum treatment, or by short aeration with air containing ammonia. The acid-insoluble lignin and in vitro cellulose digestion coefficient of treated wheat straw are shown in Table 3. The use of gas for lignin degradation has many practical features; however, the toxic effects of many gases present another problem.

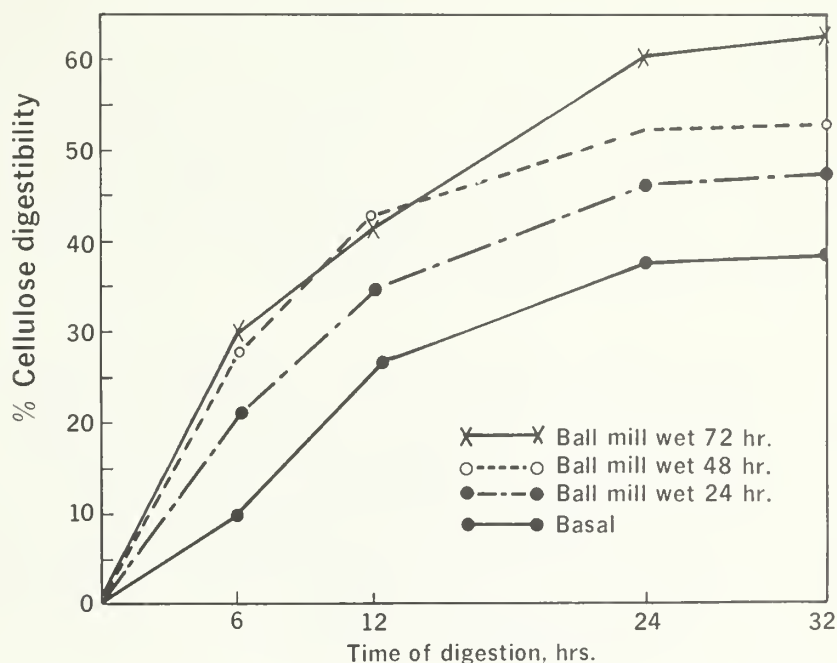


Figure 2. Effect of ball milling on cellulose digestion.

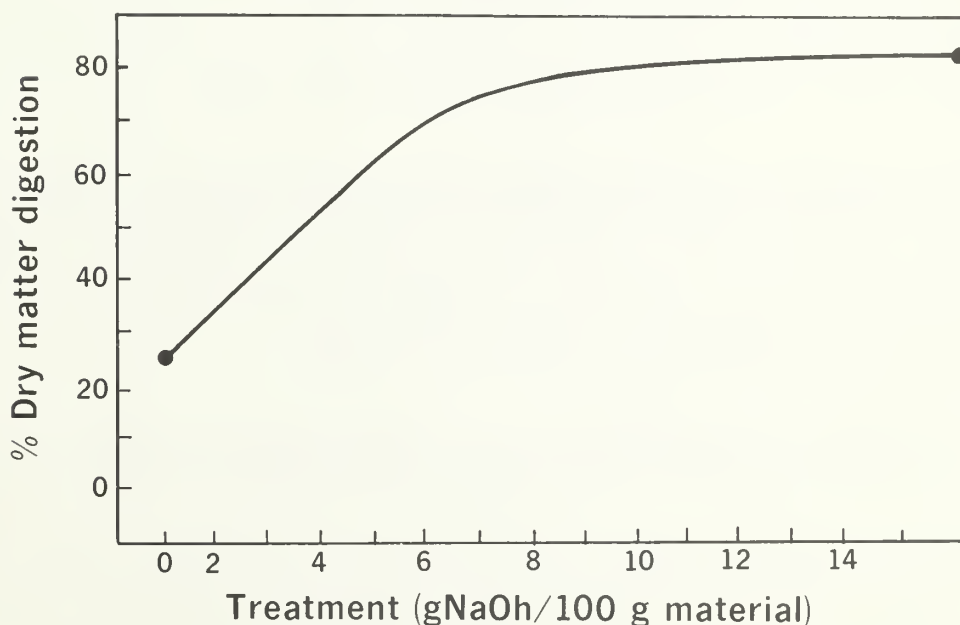


Figure 3. Effect of alkali treatment on wheat straw digestibility (Wilson and Pigden, 1964).

Table 3.--Lignin content and in vitro cellulose digestibility of wheat straw treated in 8-g. lots with ClO_2 gas (Sullivan and Hershberger, 1959)

	Acid insoluble lignin %	Digestion coefficient %
No treatment	6.7	22.8
2.5 g. NaClO_2	2.8	36.2
3.0 g. NaClO_2	3.9	24.0
3.2 g. NaClO_2	1.9	52.2

Biological treatments. The use of biological materials to degrade lignin appears to have less promise than physical or chemical procedures. However, it is not out of reason to think that an enzyme or microbial material could be developed that would act as a ligninase and degrade lignin. This would allow the cellulase enzyme to act upon the cellulose.

Summary. The problem of increasing the utilization of alfalfa stem forage lies in the area of degradation of lignin, changing its physical relationship to cellulose and hemicellulose in the plant material so that cellulase enzymes can digest the cellulose. A three-year project has recently been initiated at the Nebraska Station to study procedures for lignin degradation in alfalfa stem forage. To date only physical treatments have been studied. Ball milling is providing some promise. A review of literature indicates that of most physical procedures ball milling should show the most promise. Chemical treatments such as alkali and chlorine dioxide gas will be evaluated. They too have been shown to degrade lignin.

ADA'S RESEARCH PROGRAM

N. L. Jacobson
Iowa State University, Ames, Iowa

For some time the American Dehydrators Association has supported basic and applied research relative to alfalfa and alfalfa products. This work has contributed substantially to a better understanding of the production, composition, nutritional value, and utilization of dehydrated alfalfa. The association is to be commended for its farsighted research program and for its enthusiastic support of work recommended by its Research Council. The following is a brief review of projects supported in 1964-65 and 1965-66.

Research Supported During 1964-65

1. A study of the major nutritional constituents of dehydrated alfalfa. The study was conducted for ADA by Midwest Research Institute, Kansas City, Mo.; the analytical work was done by several laboratories under contract to MRI. This was a major research effort and involved extensive analyses on composite samples. The undertaking was top priority, supported enthusiastically by the Research Council and by ADA. Because a considerable portion of the total resources available for supporting research during 1964-65 was committed to this project, funds for allocation elsewhere were less.

The results have been published in the report, "A Study of the Major Nutritional Constituents of Dehydrated Alfalfa," submitted by the Midwest Research Institute in April 1965. The results include extensive analyses of vitamin, mineral, and amino acid contents of dehydrated alfalfa. The data provide much needed information. There has been a great demand for copies not only in the United States but in other countries.

2. Dehydrated alfalfa for dairy cattle (University of Nebraska). This project was initiated in 1963 with a tentative plan to continue it for 3 years. Initial objectives were (a) to determine the influence of dehydrated alfalfa in the concentrate mixture (grain) on performance of lactating cows, (b) to compare responses to dehydrated alfalfa supplementation in high-grain and normal-grain feeding regimes, and (c) to determine the effect of dehydrated alfalfa on the energy digestibility of a conventional dairy ration using high and normal levels of grain. In the first year, the grain mixture was not pelleted. In the second year (1964-65), the objectives were extended to include evaluation of the response to dehydrated alfalfa in pelleted grain mixtures.

A factorial design involving 36 cows each year was employed. The effects of ration on milk yield, milk fat percentage, milk solids-not-fat percentage, feed intake, efficiency of feed utilization, body weight change and certain other responses were observed. During the first year (1963-64) alfalfa hay (8 lb. per cow daily), sorghum silage (free-choice) and grain at normal and high levels were fed. The grain of half the cows at each grain level contained 10% dehydrated alfalfa. In the second experiment, 1964-65, the forages were corn silage fed free choice and medium-to-fair-quality alfalfa hay fed at a level of 5 lb. per cow daily. Nonpelleted and pelleted grain mixtures, half with 10% dehydrated alfalfa and half with none, were used.

In the first year, 1963-64, dehydrated alfalfa seemed to have a slightly depressing effect on milk production response, possibly because of the lower energy concentration in the grain mixture containing dehydrated alfalfa. The alfalfa hay was high quality and probably obscured any beneficial effect of the dehydrated alfalfa. Data for the second phase of the study, 1964-65, have not yet been summarized.

3. Methods for improving cellulose digestibility of dehydrated alfalfa in ruminants (Ohio State University). The objective was to find a way to improve the cellulose digestibility of pelleted, finely ground dehydrated alfalfa. Ten Jersey heifers were employed in the series of digestion trials to determine the effect of various ratios of corn silage and dehydrated alfalfa on the digestibility of the latter. In further experiments the effects of alfalfa hay and of beet pulp (each replacing the corn silage) were determined. It was hoped that digestibility of cellulose in the finely ground alfalfa could be enhanced by physically entrapping it in the other materials and thus cause it to be retained in the rumen for a longer period.

A small increase in dry matter and cellulose digestibilities, above that predicted on the basis of the individual components, was observed when 10% beet pulp or corn silage was fed with dehydrated alfalfa pellets. Larger proportions of these roughages did not further enhance the digestibilities (no further interaction) and alfalfa hay had very little effect at any level.

4. Research on crystalline coumestrol with identical twin calves (Purdue University). The object was to determine whether coumestrol can act as a growth stimulant for beef cattle. The procedure involved use of four sets of identical twins to determine the effect of feeding four levels of coumestrol (from 50 to 200 ppm.) on growth and efficiency of feed utilization.

5. The effect of dehydrated alfalfa meal and other dietary components on egg yolk pigmentation and reproductive performance of chickens (University of Nebraska). The objectives were (a) to determine if and how compounds present in dehydrated alfalfa affect egg production, incidence of blood spots in eggs, fertility and hatchability; (b) to compare the relative pigmenting ability of various alfalfa meals and other feeds as well as certain purified pigmenting materials; and (c) to study the physiology of egg yolk pigmentation with synthetic and natural xanthophylls.

Four dehydrated alfalfa meals (containing 17, 18, 20, and 22% protein, respectively) were fed at levels of 0, 3, 8, 13, and 18% in diets of laying hens. All diets were formulated to contain equivalent levels of protein, energy, calcium and phosphorus. There were no appreciable differences in egg production among the four alfalfa meals (varying in protein content) but production was higher for the diet containing 3% dehydrated alfalfa meal than for the others. Fertility and hatchability were essentially the same for all four meals and all dietary levels. Incidence of blood spots in this experiment was low but was highest with levels of 0 and 18% alfalfa and lowest with 3%. Xanthophyll from all four alfalfa meals was readily available for egg yolk pigmentation.

Research Supported During 1965-66:

1. Dehydrated alfalfa for dairy cattle (University of Nebraska). This is the third year of the study. The objectives are (a) to determine the influence of dehydrated alfalfa in the grain mixture on performance of lactating cows fed nonlegume roughage, (b) to compare responses to dehydrated alfalfa in a pelleted grain mixture fed according to a normal and a high-grain feeding regime, and (c) to determine the effect of supplemental dehydrated alfalfa on digestibility of rations containing normal or high levels of grain.

The roughage used is corn silage and the concentrate portion of the ration contains about 17% crude protein. Half of the animals at two levels of concentrate feeding (normal and high) receive dehydrated alfalfa as 10% of the concentrate mixture. The effects of the rations are being measured in terms of milk yield, milk fat, milk solids-not-fat, estimated net energy intake, efficiency of conversion of feed energy to milk energy, body weight changes, conception rate and health observations. A digestion trial to measure dry matter, energy, and protein digestibility also is planned.

2. Alfalfa meal as a source of unidentified growth factors for the guinea pig (University of California, Davis). The objective is to determine the active fractions or components in alfalfa meal which stimulate growth in the guinea pig. The guinea pig does not grow maximally unless a source of unidentified factor(s) is present in the diet. The best and most consistent source is alfalfa, but little is known about properties of the factor. Young guinea pigs are fed a basal diet plus various chemically derived fractions of

alfalfa meal such as water, alcohol, and ether extracts; the ash of the whole meal; and the residues resulting from each extraction procedure. The Western Utilization Research and Development Division, USDA, Albany, California is cooperating.

3. A study of methods to improve quality of dehydrated alfalfa products (University of Nebraska). Objectives are (a) to evaluate effects of commercial field management practices on yield and quality of dehydrated alfalfa, as measured by dry matter, protein, carotene, fiber, coumestrol, and nitrate content, (b) to determine the effect of insect control on yield and quality, (c) to study the relationship between field management and stand persistence and (d) to study the effect of rate of flow and drum exhaust temperatures on dehydrated alfalfa quality.

Work related to this project was initiated at the Nebraska Station in 1963. The present study, a continuation and expansion, involves management practices such as cutting at various stages of growth and cutting at different heights. Chemical determinations include carotene, protein, fiber, nitrate and coumestrol.

4. Carbohydrate metabolism in the ruminant animal as influenced by frequency of protein supplementation (New Mexico State University). The objective is to determine the effect of frequency of protein supplementation (varying from continuous to 18-day intervals) on growth and utilization of energy in the ration of sheep. The basal ration contains low-quality roughage and supplemental protein is supplied by dehydrated alfalfa pellets.

5. Effect of dehydrated alfalfa meal on the bacterial synthesis of protein from urea in cattle (Purdue University). Objectives are (a) to determine the effects of certain unidentified factors in dehydrated alfalfa meal on the bacterial synthesis of protein from urea and (b) to determine if there is any correlation between bacterial synthesis of protein and amino acid pattern in the blood.

Initially, the work involves two sets of twin steers with which digestible energy and nitrogen balance studies are being conducted. The rations are a basal and a basal plus 0.5 lb. dehydrated alfalfa meal. Other studies will involve pH, ammonia, and amino acids in rumen contents and urea nitrogen, ammonia, and amino acids in the blood. Subsequent work will involve a study of the effects of levels of dehydrated alfalfa on bacterial synthesis of protein and the relationship between protein level and unidentified factors in dehydrated alfalfa.

6. Effects of various levels of dehydrated alfalfa meal on performance and carcass quality of fattening cattle fed rations based on barley and low-quality roughage (University of Saskatchewan). The objective is to determine the effect of dehydrated alfalfa meal on rate of gain, feed efficiency, and carcass quality of fattening cattle. Twelve groups of 12 steers each are used in a factorial experiment involving three levels of dehydrated alfalfa (0, 2, 4%) and four other supplementary protein sources (basal low-protein, soybean meal, rape seed meal, urea).

Other Research Supported:

In addition, the American Dehydrators Association also has provided support for work on fiber fractions at the Western Utilization Research and Development Division, USDA, Albany, California and for digestible energy studies with swine at the University of Florida.

Each year the ADA receives a large number of requests for funds to support research on forages. The Association has been unable to support all proposals submitted but it has been wise, I believe, to select a few projects and to support them to the best of the Association's ability. In many cases the funds provided are sufficient to bring about initiation of work, which otherwise might not be started. Further, the results sometimes provide information which attracts other sources of funds for continued research. Research of a comparatively basic nature probably will have the greatest long-term significance and consequently ought to be encouraged even though the immediate application is not always apparent. However, some research of the more applied nature also warrants support since experiments of this type are needed and are less likely to receive adequate sponsorship in the future.

ALFALFA MANAGEMENT FOR DEHYDRATION

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University of Nebraska, Lincoln

Last winter at the 23rd annual convention of the American Dehydrators Association we presented our first progress report on field management of alfalfa and its relationship to quality. We will repeat some of the background we gave at that time as preparation for this second progress report.

We recognize that quality begins in the field and assume that the usual steps were taken to establish a good stand. These steps include determining soil quality and applying needed fertilizer, choosing an adapted variety, using quality seed in a properly prepared seedbed, using cultural methods of weed control supplemented with chemical weed control if needed, and using proper water management, if irrigated. After stand establishment, several field management practices are available to control quality. Process variables may also be used to control quality.

Some principles of forage management, needed to produce relatively good yields of good quality, were developed years ago from work with small plots. Principal considerations in determining time of cutting include content of specific chemical constituents, yield of chemical constituents and dry matter, digestibility and total digestible nutrients and stand persistence. Whereas older work was mainly concerned only with the effects of stage of maturity on quality, newer work is being done to harvest or process alfalfa to provide products of specific quality. Management practices which can produce the most valuable products are of primary concern, particularly those in which the processing and capital investment costs are low.

Early research. Previous research showed that dry matter yields increased with maturity; that protein content decreased while fiber increased with maturity; and that digestibility and content of digestible nutrients decreased with maturity. Proportion of leaves and stems has been repeatedly reported as possibly the most important factor in quality.

Recent research. In more recent work at the Nebraska Station using a laboratory model dehydrator, studies were made on the relationship of stage of growth to quality. Dry matter and coumestrol content increased while protein and carotene content decreased with maturity. (Analyses were part of a larger cooperative study: Hanson, Loper, Kohler, Bickoff, Taylor, Kehr, Stanford, Dudley, Pedersen, Sorensen, Carnahan, and Wilsie, "Variation in coumestrol content of alfalfa as related to location, variety, cutting, year, stage of growth, and disease," U.S. Dept. Agr. Technical Bul. 1333, 1965.)

Current research. Facilities at the Mead Field Laboratory became available in 1963 for research-demonstration studies. In our studies at this station large plots were commercially chopped and dehydrated. (The authors wish to thank Ralph and Larry Brown and their personnel of Skyline Mills, Mead, Nebr., for their cooperation in use of equipment which made these studies possible.)

Experiment M-IV was started in 1964 and continued in 1965. Five commercial management systems were imposed on a 1963 seeding of the variety Cody on the Browns' land adjacent to the station. In one system, the first cutting was made at the 10-inch stage, followed by cuttings at 1/10 bloom. In another system, the first cutting was made at full bloom, followed by cuttings at 1/10 bloom. Other systems included cutting always at one stage of growth, i.e., bud, 1/10, and full bloom. Weather conditions altered planned cutting dates to some extent. Although provisions were made to study effects of insect control on the various management systems, insect populations were never high enough to be economically important. In the accompanying tables, data are reported on a dry-weight basis. Since the last 1965 cutting was made in mid-October, statistical analyses are not completed. Only trends will be discussed. Tables 1-6 show that maximum annual yields of dry matter, carotene, and protein were obtained by cutting at 1/10 bloom. Yields per cutting were highest at full-bloom except for carotene yield, which was highest at 1/10 bloom. High quality as measured by protein and carotene content was obtained at the expense of reduced yield and of added costs of more chopping and processing.

Another phase of management research was started in 1961. Alfalfa, 36 inches tall, was cut, hand chopped into six-inch segments, and dehydrated in a laboratory dehydrator. Results presented in Table 7 show that protein and carotene contents progressively increased and fiber content decreased from the bottom to the top of the plant. The amount of dry matter was nearly constant among segments. Sixty-two percent of the proteins, 85% of the carotene, and 39% of the fiber were contained in the top half of the plant.

Since a major problem with quality exists with full-bloom alfalfa, Experiment M-III was started in 1964 and continued in 1965. Three management systems were used: (1) Standard cutting. Harvesting the entire plant at full bloom in all cuttings. (2) Multiple cutting. In the first cutting separately

Table 1.--First-year average chemical content and production for the total season. Mead Experiment M-IV. 1964.

Management	Number cuts	Dry matter lb/A	Protein content %	Protein yield lb/A	Carotene content mg/lb	Carotene yield g/A
Bud	5	8523	21.3	1816	94	796
10" - 1/10	5	8281	21.6	1785	88	728
1/10	4	9154	20.3	1858	95	868
Full - 1/10	4	8245	20.2	1660	76	630
Full	3	8682	18.8	1634	70	615

Table 2.--Relative chemical content and production (headings as in Table 1)

Bud	5	93	105	98	99	92
10" - 1/10	5	90	106	96	93	84
1/10	4	100	100	100	100	100
Full - 1/10	4	90	100	89	80	73
Full	3	95	93	88	74	71

Table 3.--Second-year average chemical content and production for the total season. Mead Experiment M-IV. 1965

Management	Number cuts	Dry matter lb/A	Protein content %	Protein yield lb/A	Carotene content mg/lb	Carotene yield g/A
Bud	5	8329	22.0	1829	114	952
10" - 1/10	5	7637	22.6	1724	114	867
1/10	5	9459	21.5	2031	110	1039
Full - 1/10	4	9170	19.9	1822	101	922
Full	4	9446	18.6	1762	83	784

Table 4.--Second-year average relative chemical content and production (headings as in Table 3)

Bud	5	88	102	90	104	92
10" - 1/10	5	81	105	85	104	83
1/10	5	100	100	100	100	100
Full - 1/10	4	97	93	90	92	89
Full	4	100	87	87	75	75

harvesting the top and bottom halves at full bloom and harvesting the entire plant at full bloom in other cuttings. (3) Partial cutting. At full bloom in the first cutting harvesting the top half, leaving the stubble (bottom half) in the field, harvesting the entire plant at an early stage (late bud in 1964, pre-bud and bud in 1965) in the second cutting, and harvesting the entire plant at full bloom in other cuttings.

Results presented in Tables 8-13 show that with the multiple cutting system, dry matter, protein and carotene yields were reduced as compared to the standard cutting system. All of this loss appeared in the first cutting and was

due to trampling of the forage, particularly in opening the plot when cutting the top half. Part of this loss may not occur in commercial operations. Use of a front-mounted rather than a side-mounted chopper would reduce losses especially on plot work.

Table 5.--Two-year average chemical content and total season production.
Mead Experiment M-IV. 1964-1965

Management	Number cuts	Dry matter lb/A	Protein content %	Protein yield lb/A	Carotene content mg/lb	Carotene yield g/A
Bud	5	8426	21.6	1823	104	874
10" - 1/10	5	7959	22.0	1755	100	797
1/10	4.5	9307	20.9	1945	102	954
Full - 1/10	4	8708	20.0	1741	89	776
Full	3.5	9065	18.7	1698	77	699

Table 6.--Two year average relative chemical content and total season production (headings as in Table 5)

Bud	5	91	103	93	102	92
10" - 1/10	5	86	105	90	98	84
1/10	4.5	100	100	100	100	100
Full - 1/10	4	94	96	90	87	81
Full	3.5	97	89	87	75	73

The two-year averages show that the partial cutting system gave increased protein and carotene contents and carotene yield over the standard cutting system. Regrowth in the stubble was from the crown and axillary buds. Dry-matter yields of the partial cutting system for 1965 and the two-year average were less than the standard cutting system; however, this was to a great extent caused by the September 1965 rainy period in which one cutting in the partial cutting system was lost.

Table 7.--Chemical content by 6-inch segments. First cutting. 1961

Segments in inches	Protein content %	Carotene content mg/lb	Fiber content %	Dry matter g
30-36 (top)	22.0	92	23	128
24-30	20.3	82	29	135
18-24	16.9	50	36	132
12-18	13.6	21	44	127
6-12	12.3	11	47	129
0-6 (bottom)	11.7	9	49	129
Entire plant	14.9	48	37	780

Results presented in Tables 14-19 show that a high-quality product may be obtained on full-bloom alfalfa in the first cutting by cutting the top and bottom halves of the plant separately. The two-year average protein and carotene contents of the top half were respectively, 22 and 60% higher and the fiber content 29% lower than those of the entire plant in the first cutting.

Table 8.--First-year average chemical content and production for the total season. Mead Experiment M-III. 1964

Management	Cutting	Dry matter lb/A	Protein content %	Protein yield lb/A	Carotene content mg/lb	Carotene yield g/A
Full bloom Entire plant	1,2,3	7085	16.9	1195	51	361
Full bloom Top vs. bottom Entire plant	1, 2,3	6473	17.1	1109	50	322
Full bloom, top Bud, entire FB, entire	1, 2, 3,4	7758	19.2	1491	67	519

Table 9.--First year average relative chemical content and production (headings as in Table 8)

Full bloom Entire plant	1,2,3	100	100	100	100	100
Full bloom Top vs. bottom Entire plant	1, 2,3	91	101	93	98	89
Full bloom, top Bud, entire FB, entire	1, 2, 3,4	110	114	125	131	144

Table 10.--Second-year average chemical content and production for the total season. Mead Experiment M-III. 1965

Management	Cutting	Dry matter lb/A	Protein content %	Protein yield lb/A	Carotene content mg/lb	Carotene yield g/A
Full bloom Entire plant	1,2,3,4	9725	19.1	1861	72	698
Full bloom Top vs. bottom FB, entire	1, 2,3,4	8899	19.5	1739	76	673
Full bloom, Top Prebud, entire FB, entire	1, 2, 3,4	7277	20.2	1467	85	619
Full bloom, top Bud, entire FB, entire	1, 2, 3,4	7641	20.2	1545	91	696

Some dehydrators have cut and windrowed alfalfa for a few hours before dehydrating to help remove some of the water. We conducted an experiment to determine what happened to the common nutrients with this method of harvesting. Figure 1 shows some of the results of this experiment. We chopped forage from windrows after several drying time intervals. Fresh forage was chopped at

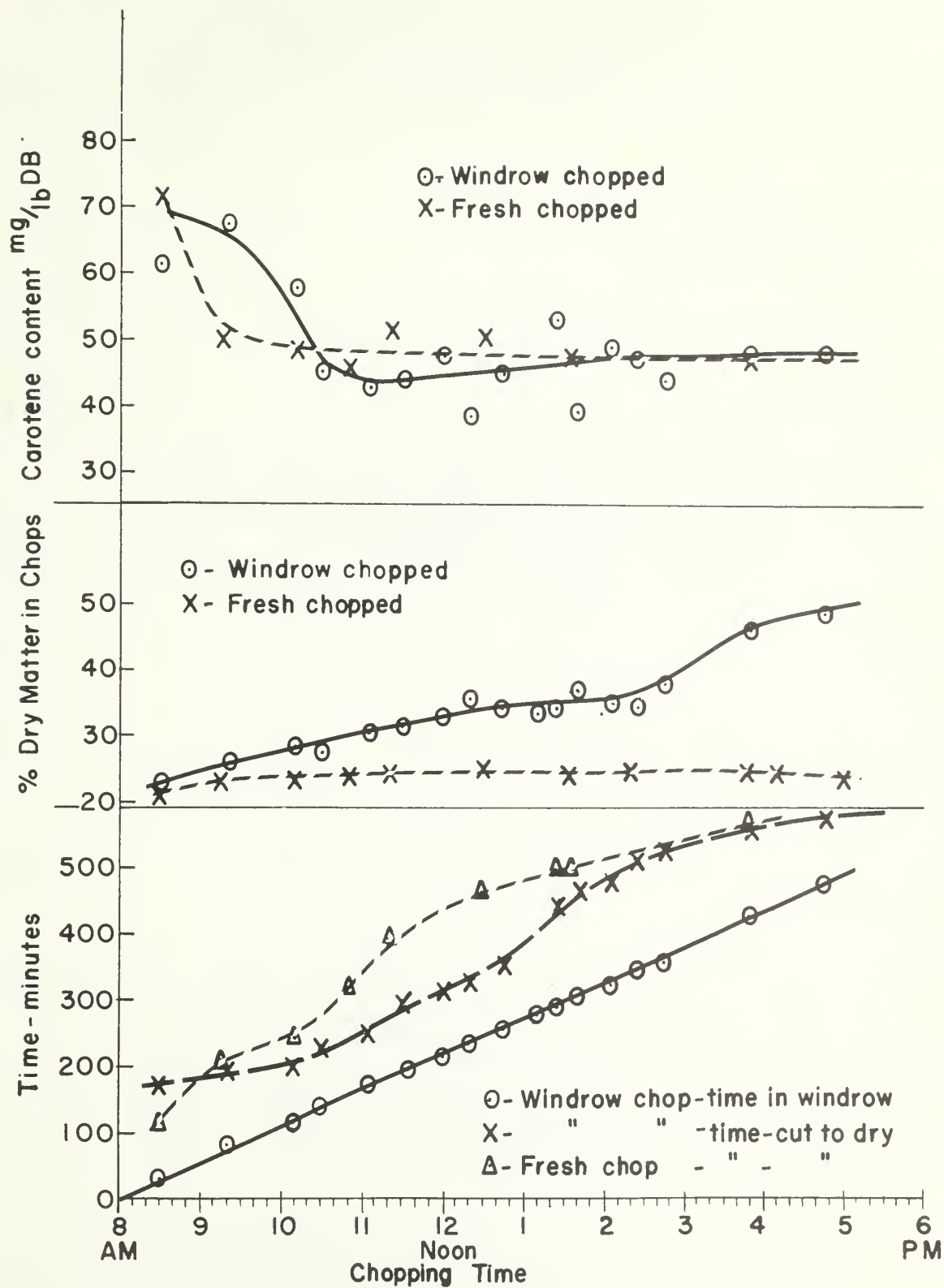


Figure 1. Effects of partial windrow drying.

Table 11.--Second-year average relative chemical content and production for the total season. Mead Experiment M-III. 1965

Management	Cutting	Dry matter	Protein content	Protein yield	Carotene content	Carotene yield
Full bloom	1,2,3,4	100	100	100	100	100
Entire plant						
Full bloom						
Top vs. bottom	1,	92	102	93	106	96
FB, entire	2,3,4					
Full bloom, top	1,					
Prebud, entire	2,	75	106	79	118	89
FB, entire	3,4					
Full bloom, top	1,					
Bud, entire	2,	79	106	83	126	100
FB, entire	3,4					

Table 12.--Two-year average chemical content and total season production. Mead Experiment M-III. 1964-1965

Management	Cutting	Dry matter	Protein content	Protein yield	Carotene content	Carotene yield
		lb/A	%	lb/A	mg/lb	g/A
Full bloom	1,2,3,4	8405	18.2	1528	63	529
Entire plant						
Full bloom						
Top vs. bottom	1,	7686	18.5	1424	65	498
Entire plant	2,3,4					
Full bloom, top	1,	7517	19.7	1479	76	569
Prebud, entire	2,					
FB, entire	3,4					
Full bloom, top	1,	7699	19.7	1518	79	608
Bud, entire	2,					
FB, entire	3,4					

intervals through the windrow test period. Several comparisons between windrow and fresh-chopped forage can be made. Protein and fiber contents were not appreciably altered by windrow-chopping. The carotene content of windrow-chopped forage was reduced about one-third from the initial content but this loss was paralleled by regular chop. We are not ready to advocate that this is a good management practice, but plan to continue this investigation. In a single experiment using sprinkler irrigation we found that water-soluble components in fresh forage may be reduced during rainfall.

Summary. Two-year results from management experiments with alfalfa involving growth stages harvested and processed by commercial dehydration equipment showed that maximum annual yields of dry matter, protein, and carotene were obtained when cut at 1/10 bloom even though maximum yields of dry matter and protein per cutting were obtained at full bloom.

Table 13.--Two-year average relative chemical content and total season production. Mead Experiment M-III. 1964-1965

Management	Cutting	Dry matter	Protein content	Protein yield	Carotene content	Carotene yield
Full bloom	1,2,3,4	100	100	100	100	100
Entire plant						
Full bloom						
Top vs. bottom	1,	91	102	93	103	94
Entire plant	2,3,4					
Full bloom, top	1,					
Prebud, entire	2,	89	108	97	121	108
FB, entire	3,4					
Full bloom, top	1,					
Bud, entire	2,	92	108	99	125	115
FB, entire	3,4					

Table 14.--First-year average chemical content and production for the first cutting. Mead Experiment M-III. 1964

Management	Dry matter	Protein content	Protein yield	Carotene content	Carotene yield
	lb/A	%	lb/A	mg/lb	g/A
Full bloom	3492	18	631	43	151
Entire plant					
Full bloom					
Top half	1341	22	301	70	94
Bottom half	1688	15	254	30	50

Table 15.--First-year average relative chemical content and production for the first cutting (headings as in Table 14)

Full bloom	100	100	100	100	100
Entire plant					
Top half	44	122	54	163	65
Bottom half	56	83	46	70	35

Table 16.--Second year average chemical content and production for the first cutting. Mead Experiment M-III. 1965

Management	Dry matter	Protein content	Protein yield	Carotene content	Carotene yield	Fiber content	Fiber yield
	lb/A	%	lb/A	mg/lb	g/A	%	lb/A
Full bloom	4122	17.9	738	55	233	36.6	1508
Entire plant							
Full bloom							
Top half	2521	21.7	546	86	216	29.0	730
Bottom half	798	13.1	104	30	24	44.6	356

Table 17.--Second-year average relative chemical content and production for the first cutting. Mead Experiment M-III. 1965

Management	Dry matter	Protein content	Protein yield	Carotene content	Carotene yield	Fiber content	Fiber yield
Full bloom	100	100	100	100	100	100	100
Entire plant							
Full bloom							
Top half	76	121	84	156	90	79	67
Bottom half	24	73	16	55	10	122	33

Table 18.--Two-year average chemical content and production for the first cutting. Mead Experiment M-III. 1964-1965

Management	Dry matter	Protein content	Protein yield	Carotene content	Carotene yield	Fiber content	Fiber yield
	lb/A	%	lb/A	mg/lb	g/A	%	lb/A
Full bloom	3807	18.0	684	50	192	35.7	1359
Entire plant							
Full bloom							
Top half	1931	22.0	424	80	155	25.3	488
Bottom half	1243	14.4	179	30	37	42.2	525

Table 19.--Two-year average relative chemical content and production for the first cutting. (headings as in Table 18)

Full bloom	100	100	100	100	100	100	100
Entire plant							
Full bloom							
Top half	61	122	70	160	81	71	48
Bottom half	39	80	30	60	19	118	52

Multiple cutting of full-bloom alfalfa, in which the top and bottom halves of the plant were harvested and processed separately, effectively separated forage into high and low-quality products. Partial cutting of full-bloom alfalfa, in which in the first cutting the top half was harvested leaving the bottom half in the field, the entire plant was harvested at prebud stage in the second cutting and at full bloom in other cuttings, gave improved total season quality. The use of multiple cutting and partial cutting systems can lead to improved quality products without additional capital investment.

Partial windrow drying may cut costs of production without lowering protein content.

COUMESTROL AND OTHER PHENOLICS IN ALFALFA

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Our Division, USDA's Agricultural Research Service, has been actively engaged in the problem of plant estrogens as growth factors for a little over 10 years, and I am not able to say at this point that we have all the answers yet. In looking over the proceedings of previous conferences, I was surprised to find that members of our staff or of cooperating laboratories have presented progress reports in 14 talks during the 6 technical alfalfa conferences since 1955. My presentation today represents our 15th report to you on this subject. Today, I should like to summarize the findings reported earlier and also bring you up-to-date on developments since the Eighth Conference which was held in February 1963.

The first progress report, entitled "Estrogenic Factors in Alfalfa" was presented at the Third Technical Alfalfa Conference, held in July 1955. Dr. Floyd DeEds, then Chief of our Division's Pharmacology Laboratory, was instrumental in setting up this project and presented this first report.

By way of introduction, he pointed out that certain forage crops, particularly legumes, are associated with two seemingly diverse phenomena, increased growth rate and feed efficiency and also serious reproductive disorders which often result in loss of fertility, stillbirth, or early death of the young. He suggested that plant estrogens in forage might account for both of these effects and reported that earlier workers had noted isoflavonoid compounds in subterranean and red clover, and that serious reproductive problems in sheep had been attributed to these compounds, which were shown to have estrogen-like properties when tested on rats and mice.

At that 1955 meeting, he reported that our Division had begun a systematic attack on isolation and identification of chemical compounds responsible for estrogenic activity of alfalfa and ladino clover. He then described the bioassay procedures that we were employing for following the steps in the isolation process. These involved feeding either the whole forage or isolated fractions to immature female mice and determining the increase in uterine size. At the time of this report, we had already been working on this project for several months and had achieved considerable concentration of estrogenic activity, but we had not yet isolated any of the estrogenic compounds, nor did we yet have any idea of their structures.

At the Fourth Technical Alfalfa Conference, held at Albany, Calif., in July 1957, I discussed the occurrence of estrogens in forage crops. During the two previous years, we had been obtaining from dehydrators throughout the country as many samples as they could supply. The estrogenic content was extremely variable, ranging from none in some to extremely high in others. A 10-mg. uterine response is a control level.

During this time we also began growing a small stand of alfalfa which we sampled and analyzed periodically. We found that estrogenic content varied

markedly with stage of maturity and from cutting to cutting, as well as at different seasons. Further, the results from year to year were not consistent. There seemed to be an environmental factor or factors influencing estrogen buildup in the plant. From a cooperative study on ladino clover undertaken in cooperation with the Crops Research Division of USDA's Agricultural Research Service, we obtained a suggestion that one of these factors might be disease.

In the 1957 harvest, virus-infected Louisiana 6 was the only one of the six clonal lines studied that contained considerable estrogenic activity. The corresponding virus-free sample of Louisiana 6 was very low in activity. We reported at that time that the high level of activity in the former sample suggested a possible correlation between estrogenic activity and virus infection. To follow up this lead, we carried out a more detailed study of the relation between virus infection and estrogen buildup during the 1958 season and again found a correlation between virus infection and estrogen increase in certain cases. However, this second year the virus-free samples of the La 6 clone now contained more estrogen than the virus-infected material. This confused the picture and it remained for Drs. Hanson and Loper, several years later, in a series of excellent experiments, to clearly establish that disease is the predominant factor causing high estrogen levels in the plant. Their work will be discussed later in this talk.

At the Fifth Technical Alfalfa Conference in August 1958, I reported a number of related observations on reproductive failure, possibly attributable to plant estrogens. Serious infertility has been reported from Australia and USA with sheep, and in Israel, Germany, and USA among cattle. Elsewhere, disturbances in reproductive functions have been reported. Estrogenic activity in forage pasture has been reported from at least 12 countries. It may well be that numerous instances of unexplained breeding difficulties could have been attributed to the natural estrogen present in the feed if this relationship had been better recognized.

The exact mechanism of infertility is not fully understood. Recent work has shown that interference with spermatozoan transport through the genital tract is an important factor, but abnormal formation and transport of ova and interference with implantation are probably also involved.

I also reviewed work that suggested a growth-promoting effect. In 1957, workers at Iowa State University reported limited experiments in feeding the purified plant estrogen, genistin, to fattening lambs and obtained measurably increased growth rates. The addition of pure genistin to the diet resulted in a 15% increase in rate of gain.

At this same Conference, Professor Matsushima, who was then at the University of Nebraska, reported positive evidence of weight-gain stimulation in steers fed alfalfa naturally high in estrogenic activity. A daily intake of 5 pounds of this alfalfa produced the same daily weight gain in pounds (2.46) as did the addition of stilbestrol to the diet (2.47). When stilbestrol and dehydrated alfalfa were combined as a supplement, the rates of gain were better than when either was used alone.

Professor Matsushima continued these studies in cooperation with us, and in 1961, at the Seventh Technical Alfalfa Conference, he presented additional findings. His 1960 feeding trial was designed to compare feeding values of dehydrated alfalfas with low and high estrogen potencies. His results showed no difference. However, his results were complicated by the fact that little or no difference was obtained by feeding stilbestrol. In his 1961 trials, dehydrated meals containing three levels of estrogen were used. Again, no significant difference in gain or feed efficiency was noted among the groups of cattle. The three levels fed were 25, 100, and 250 ppm. However, this time the steers fed 10 mg. stilbestrol gained significantly faster than those without this additive, regardless of estrogen level.

At the Sixth Technical Alfalfa Conference held in July 1959, my presentation was entitled "Chemistry and Distribution of Estrogens in Forages." By this time we had ascertained the structure of coumestrol. We had also found that alfalfa contains an isoflavone, daidzein, also estrogenic, which had not been previously reported in forages. This brought to four the number of estrogenic isoflavones found in forages. We then studied the relative amounts of these five estrogens in four typical forages. Daidzein was found in all four forages. Biochanin A, previously reported in red and subterranean clover was also found in alfalfa. Coumestrol was found in all forages studied, including bur, strawberry, and white clover. Because of their relatively greater abundance, the isoflavones account for most of the estrogenic activity in red and subclover. However, the contribution of the isoflavones to the estrogenicity of alfalfa and ladino clover is insignificant and coumestrol is the more important estrogen in these two forages. I also reviewed the structures of the natural animal estrogens and tried to show the structural relationship between them and stilbestrol and the plant estrogens.

Although there is strong circumstantial evidence that the pure compounds, coumestrol, genistein, daidzein, formononetin, and biochanin A, are active in sheep and cattle, so far they have been found to have estrogenic activity only in mice. The lack of such studies is related to the high cost of preparation of these compounds in sufficient quantity because their activity relative to stilbestrol is so low. However, a recent report has stated that a study with genistein and biochanin A is under way and preliminary results suggest that both are estrogenically active in sheep.

Also at the 1959 conference, Dr. Booth of our Pharmacology Laboratory presented a paper entitled "Past, Present, and Future of Estrogens in Feeds." He described the early work on stilbo and presented some data on feeding trials, showing that many instances are reported in the literature where stilbo produced no growth benefit. Dr. Booth suggested that perhaps the basal ration already contains a sufficient level of plant estrogens to enhance growth in those instances where no response to added stilbestrol was observed. However, he pointed out that it had yet to be established whether or not the plant estrogens could substitute for stilbestrol as a growth promoter. He described a number of large-animal studies being undertaken in cooperation with several interested animal husbandry groups. We were undertaking to obtain and furnish both high- and low-estrogen meals for these experiments.

The results of some of these animal feeding trials were presented in a symposium on coumestrol, at the following meeting, the seventh conference, held in July 1961. I have already mentioned the report that Professor Matsushima presented. Dr. Elam of the Beef Cattle Research Branch of Agricultural Research Service, USDA, also reported his findings. He compared digestibility and nitrogen utilization of high and low coumestrol alfalfa and could find no difference between them in nitrogen retention. In later studies, employing pure crystalline coumestrol, he again obtained negative results for increased nitrogen retention.

Professor Oldfield presented a paper at this symposium entitled "A Review of Oregon Research on Estrogens and Forages in Sheep Feeding" covering several years of feeding trials. In his 1959 and 1960 trials, the high-estrogen meals gave better growth than the low-estrogen meals. In his third trial, a coumestrol concentrate obtained by acetone extraction of dehydrated alfalfa was employed, which contained over 3000 ppm. of coumestrol. It was added to the control to give a series of fortified diets containing coumestrol contents ranging from 18 to 152 ppm. Although a slight increase was obtained with the two higher levels, this difference was not statistically significant. In work subsequent to this symposium, crystalline coumestrol added to a control diet again produced no added gain over the control. However, it is interesting that organoleptic tests consistently showed improved tenderness and juiciness scores for lamb roasts from animals fed high-coumestrol diets. Professor Beeson did not present a paper at this symposium. However, he reported in a Purdue Agricultural Experiment Station Bulletin that he was not able to find a growth-promoting effect due to coumestrol. In fact, he reported that daily gains of steers fed dehydrated alfalfa pellets containing a low level of coumestrol were significantly greater than those of steers fed pellets containing a high level. He further found that feeding 5 mg. of stilbestrol and low-coumestrol dehydrated alfalfa pellets produced significantly greater gains than any other combination of oral stilbestrol and coumestrol. We have supplied Professor Beeson with additional quantities of coumestrol and he is now repeating this experiment to ascertain if he is able to confirm these results. However, Professor Beeson presented a paper entitled "Growth Factors in Dehydrated Alfalfa Meal for Beef Cattle" at the eighth conference in which he presented additional evidence of a factor in dehydrated alfalfa meal which improves the feed efficiency in beef cattle. It is possible that this factor may not be related to plant estrogens.

Another symposium paper was that of Mr. Livingston of our laboratory. He described methods that we had developed for the quantitative determination of coumestrol in fresh and dried alfalfa. The procedure involves a combination of paper chromatography and fluorometry. Advantage is taken of the intense fluorescence of coumestrol under ultraviolet light to read the samples directly on the developed chromatogram. Since these methods were rapid and fairly accurate, they permitted us to undertake several research projects, in which the numbers of assays ran into the thousands.

One of these involved a cooperative undertaking with the Crops Research Division of USDA's Agricultural Research Service. Dr. Hanson, who presented the paper this morning on "Varietal Improvement of Alfalfa" was the project leader. He arranged to have several varieties of alfalfa grown under known conditions at seven different experiment stations throughout the country. Periodically, the alfalfa was sampled and sent to us for coumestrol analysis.

Dr. Hanson presented a progress report at the 1961 symposium. The studies indicated that variation in estrogen content appeared to be caused predominantly by environmental factors. Variety was much less important. Subsequently, Dr. Hanson established that the predominant factor influencing coumestrol content is the presence or absence of disease. A diseased plant produced much more estrogen than a healthy plant. This confirmed our earlier findings with virus infection and tended also to explain the earlier findings of progressive buildup of estrogen as the plant matured, since the possibility of infection would be expected to increase concurrently with age of plant. In certain dry areas such as Utah and parts of California, the plants are relatively disease-free and the coumestrol content remains quite low throughout the growing cycle.

The results of some of Dr. Hanson's studies in which comparable plants are grown with and without disease are very dramatic. Disease-free alfalfa can be expected to be low in coumestrol regardless of age or stage of plant growth. Infecting alfalfa foliage with either of two leaf-spot pathogens, Pseudopeziza medicaginis or Leptosphaerulina briosiana, results in a rapid increase in coumestrol.

These findings suggest that alfalfa forage with poor quality because of infection by one of the pathogens is likely to have increased estrogenic properties. Likewise, good forage, free of disease, is likely to have little. This relationship between foliar diseases, coumestrol content, and alfalfa forage quality needs further investigation.

At the Eighth Technical Alfalfa Conference held in Colorado Springs in 1963, two papers discussed the estrogen work. Professor Oldfield presented a progress report on his growth studies with high and low estrogen diets, which I have already discussed together with his earlier report.

Dr. Kohler described cooperative work of our laboratory with a number of dehydrator operators, which enabled us to obtain 75 tons of high-coumestrol alfalfa for feeding tests, and for preparation of high-coumestrol concentrates and ultimately, for the preparation of 2500 grams of pure crystalline coumestrol. The estrogen content of the commercially grown alfalfa was determined during the period of plant growth, and it was arranged that the material would be harvested at the peak estrogen level, even though this would be well beyond the normal harvest period. As the alfalfa matured, the estrogen content rose until a very high value was obtained, well beyond the normal harvest period.

As an extra dividend, from this study we were able to obtain a large supply of a number of other phenolic compounds associated with coumestrol in these meals. During the period since our last report, we have been engaged in determining their structures. A number are coumestans closely related to coumestrol. Others are flavones, not previously found in forages. This characterization work is continuing.

Summary. The possibility that coumestrol might have growth-promoting properties certainly does not look as bright as we had hoped. However, we plan to continue these studies. We have contracted with a commercial company to prepare 6 kg. of crystalline coumestrol which we hope will be sufficient to provide the definitive answer on the role of coumestrol in animal nutrition.

THE NATURE OF THE XANTHOPHYLL PROBLEM

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Two years ago, I was asked to present a paper at the annual convention of the American Dehydrators Association on "Xanthophylls--What They Are and How Do You Measure Them?" At that time, we had made a survey of methods in use and had some comparative results on identical samples. No two laboratories used identical methods and the spread between results at different laboratories was dismaying. An obvious need existed for agreement on a method which could serve as a basis for contracts between buyer and seller.

This problem is not too difficult, since meticulous attention to details of procedure can lead to reproducible results. But the user of alfalfa is not interested only in a number. He needs to know how much pigmenter he has to add to the ration in order to obtain a desired level of color in poultry products. The percentage of xanthophylls fed which is deposited in the skin of broilers or the yolk of eggs (literature reports indicate 35 to 78%) is a function of at least three factors.

First, xanthophyll is complex and quantitative knowledge of pigmentation potency of individual components is lacking. Pigmentation potency, as I define it, is a function of both integrated light absorption of the specific pigments and the percentage deposition of individual pigments. Second, methods of analysis for xanthophylls are empirical compromises and measure different proportions of all of the pigments of the complex. By altering analytical conditions, it is possible to raise or lower the apparent xanthophyll content of a sample. Of course such changes would in turn change the apparent percentage biological availability based on the chemically determined amount of xanthophylls fed. Third, it seems possible that cell-wall structures or chloroplast structure may interfere with emulsification of xanthophylls by bile salts in the digestive tract, and xanthophylls may differ in emulsifiability and in transfer across cell membrane. Such interference might make xanthophylls of alfalfa less available than corn or other xanthophylls.

We have been working on all three aspects of the problem at the Western lab in Albany, Calif., in cooperation with research workers at the South Dakota Experiment Station, Purdue University, Archer-Daniels-Midland, Ralston Purina, Hoffman LaRoche, Abbott Laboratories, and others. Our overall objectives at the Western lab are to develop means of chemically measuring pigmentation potency which will permit the alfalfa processor to sell a product of uniform predictable effectiveness and further, to improve, if possible, overall pigmentation potency of alfalfa meal by developing new or improved processes for its manufacture. The material of this symposium is presented as a definition of the problems and a report of progress rather than as a solution.

First, I will discuss the problem of different alfalfa xanthophylls and their pigmentation potency. The most comprehensive work done, I believe, on alfalfa xanthophylls was carried out at the Western Laboratory in 1954 (J. Agr. Food Chem. 2:563). It was found that five main xanthophylls of fresh alfalfa

constitute about 99% of the total xanthophyll content. The structures are shown in Figure 1. Of these, lutein, violaxanthin, and neoxanthin account for over 90%. Lutein is the largest single component and constitutes over 40% of the total. Considerable work is reported which indicates that lutein and zeaxanthin are found in eggs and skin (Bunnell and Bauernfeind, Prox. XI World's Poultry Congress, Mexico City, 1958; Tagwerker *et al.*, Proc. XII World's Poultry Congress, Sydney, 1962). Marusich *et al.* have reported that violaxanthin is not an effective pigmenter for eggs (Poultry Sci. 39:1338, 1960). There is evidence, however, that pigmentation potencies for egg yolk and skin are not closely correlated so we plan to reinvestigate the entire question of violaxanthin potency. Insofar as I know, no one has specifically tested neoxanthin or cryptoxanthin for either type of pigmentation potency. Further, no rigorous quantitative data on relative potencies are available for any of the pigments.

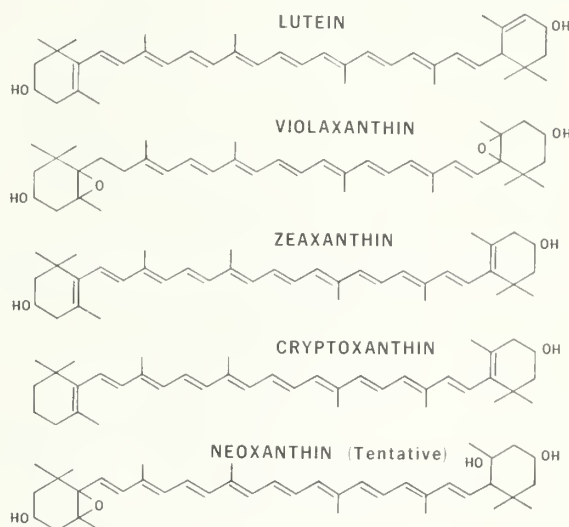


Figure 1. Structures of the five main "Xanthophylls" of fresh alfalfa.

As I mentioned, the five main xanthophylls shown in Figure 1 were found in fresh alfalfa. When alfalfa is dehydrated the picture becomes much more complicated. As the earlier work showed over 40 xanthophyll components are present in the dried products. These are for the most part *cis*-isomers of the main five, which apparently occur in the all-*trans* form in the fresh alfalfa. For example, we have identified three *cis*-isomers of lutein in dehydrated alfalfa extracts by comparing them on thin layer chromatography (TLC) with the isomers formed from pure crystalline lutein by means of iodine isomerization. In the case of violaxanthin however, it appears that large losses occur in addition to *cis*-isomerization, since the amounts of violaxanthin-like compounds in dehydrated alfalfa extracts seem low in comparison with fresh alfalfa.

Our approach to the problem of multiplicity of xanthophylls is to isolate large amounts of the five all-*trans* compounds from fresh alfalfa and to prepare the corresponding isomers by iodine isomerization and subsequent isolation. Large-scale extraction and chromatography are used with thin-layer chromatography (TLC) as a monitoring technique. Thus far we have isolated more than two grams of pure all-*trans* lutein and are attempting to prepare the needed amounts of the remaining four chief pigments. This will permit a bioassay of each plus its iodine-isomerized equilibrium mixture.

So much for this phase of the problem. Next I should like to refer briefly to the problem of analytical methodology. Lacking information on which compounds produce how much pigmentation, we feel that the analytical procedure which should be used temporarily, is one which includes all of the natural pigments of alfalfa but which excludes, as much as possible, artifacts produced by oxidation, e.g., polyoxycarotenoids more polar than neoxanthin. Procedures available up to the present time have not been designed to make such a cut-off. Further, chlorophyll degradation products have contaminated xanthophyll fractions during chromatography and have caused high results. Mr. Livingston of our laboratory and Dr. Quackenbush of Purdue University will discuss progress on analytical problems in subsequent presentations.

The third problem to which I referred is bioassay for pigmentation potency and upgrading of alfalfa from that standpoint. Our work on chicks is done on day-old or 7-14-day depleted chicks on soy-milo types of rations using three or more levels in a geometric progression of dosages. This procedure is being used to determine the pigmentation potency of isolated individual xanthophylls mentioned above, to determine the effectiveness of analytical procedure fractions, and to determine the effect of process steps and variables on pigmentation potency. Dr. Quackenbush will report some similar work done in his laboratory. Dr. Carlson and Dr. Sullivan will discuss work on yolk pigmentation later in this symposium. I should like next to show you data (Figure 2).

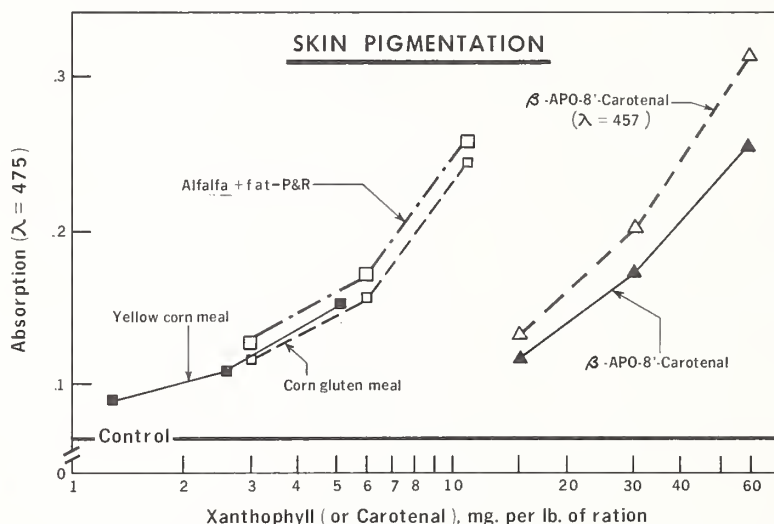


Figure 2. Chick pigmentation study showing the relative pigmentation potency of alfalfa, corn gluten meal, yellow corn meal and β -apo-8'-carotenal.

that show the comparison of β -apo-8'-carotenal with alfalfa xanthophyll. β -Apo-8'-carotenal is much more poorly absorbed than alfalfa carotenoids. The data in Figure 2 also show that alfalfa xanthophylls as determined by our present interim procedure are absorbed as well as the xanthophylls of corn or corn gluten meal. In Figure 3 are shown some recent results which show that amazingly high losses of xanthophylls are suffered during the dehydration process. Further, these losses are highly responsive to changes in outlet temperature but not to inlet temperature. At the same time carotene losses were very slight. Therefore the ratio of xanthophyll to carotene varies tremendously--from 2.3 to 1 in fresh alfalfa to 1.2 to 1 in the same alfalfa dehydrated with

the outlet temperature of our pilot plant dehydrator at 300°F. How many people are using carotene analyses multiplied by a factor in order to "estimate" xanthophyll content? We used to use a factor of 1.6 to 1. Some use 2 to 1. Now we know that this approach is worthless.

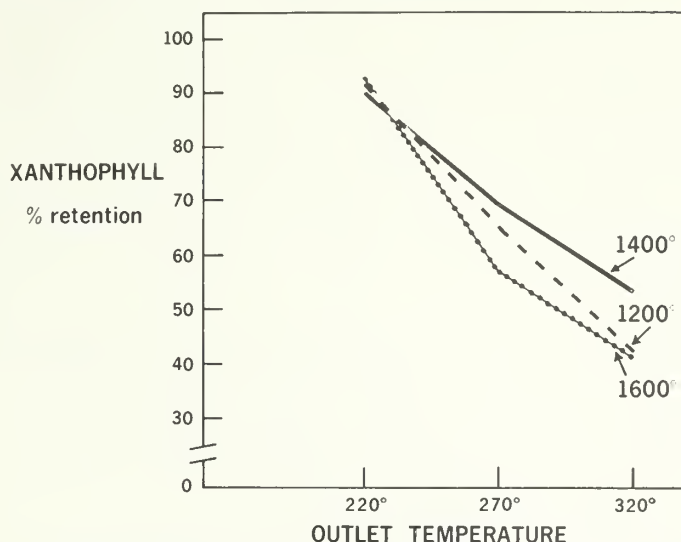


Figure 3. Dehydration experiment showing the effects of inlet and discharge temperatures on xanthophyll content of the products.

In bringing my introductory remarks to a close, I should like to suggest that there are clearly some difficult problems which are unsolved. But in working on their solution we are likely to learn much on how to improve the dehydrated alfalfa product. We have already found two important leads to such improvement, namely, increasing the biological availability of xanthophylls by pelleting as will be discussed by Dr. Carlson and second, increasing xanthophyll content of dehydrated alfalfa by control of dehydration outlet temperatures.

XANTHOPHYLL ANALYSIS IN RELATION TO PIGMENTATION

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In my laboratory we have been interested in xanthophyll analysis for many years. The recent increased interest in dietary components which are capable of increasing the color of egg yolks and of the skin of broilers has placed a new emphasis on the need for a reliable method of analysis for xanthophylls in alfalfa meal and in corn and its byproduct. We have taken the view that if we are to have a realistic method for xanthophyll analysis, we must (a) know the relative pigmenting potencies of the different individual pigments and (b) develop means of separating the potent pigments from the inactive pigments.

Accordingly, we began work several years ago to isolate the individual carotenols and to compare their relative skin-pigmenting potencies with young

chicks under controlled conditions. The individual carotenols were prepared by chromatographic separation of extracts from natural sources. A corn gluten extract ("xanthophyll oil") was fractionated essentially according to the procedure of Quackenbush *et al.* (*J. Agr. Food Chem.* 9:132, 1961) to provide zeaxanthin, zeinoxanthin, cryptoxanthin, and neolutein. Alfalfa meal (sun-cured) was extracted to obtain lutein and a fraction designated as "polyoxy pigments" which was eluted from a magnesia column after removal of zeaxanthin. This fraction was recognized to be a mixture and was tested without attempts to effect further separation. All of the individual pigments to be tested were rechromatographed until homogenous and each showed its characteristic spectral absorption curve (Cary). Commercial β -carotene (Nutritional Biochemicals), β -apo-8'-carotenal and canthaxanthin (both synthetic, Hoffman-LaRoche) were chromatographed before use.

The individual pigments were fed to male White Rock chicks, as supplements to a diet consisting chiefly of white corn and soybean meal. The details of the experiments have been described elsewhere (Quackenbush *et al.*, *J. Assoc. Offic. Agr. Chemists*, in press). The pigments were fed for a period of 4 or 5 weeks; then analyses were made of the skin from the toe web and the shank to determine the amount and kinds of pigment present.

Results with purified pigments. The data show that the carotenols differ widely in their pigmenting efficiency (Table 1). In each of the two series of

Table 1.--Pigmenting effect of individual carotenoids

Series	Pigment <u>1/</u>	Type of pigment	Pigment consumed (C)	Pigment in skin (T)	Ratios <u>2/</u>	
			mg/bird	$\mu\text{g}/100 \text{ cm}^2$ of surface	F/T	T/C
A	None	--	--	6	--	--
	Zeaxanthin	Dihydroxy	19	67	.90	3.4
	Cryptoxanthin	Hydroxy	18	51	.90	2.9
	Zeinoxanthin	Hydroxy	18	25	.80	1.4
	β -apo-8'-carotenal	oxy	19	34	.55	1.8
	Canthaxanthin	oxy	25	81	.90	3.3
B	None	--	--	11	--	--
	β -Carotene	Hydrocarbon	20	12	0.07	0.6
	Zeaxanthin	Dihydroxy	20	76	0.85	3.8
	Lutein	Dihydroxy	21	76	0.94	3.6
	Neolutein	Dihydroxy	21	65	0.93	3.0
	Polyoxy pigments	oxy	19	15	--	0.8
	β -Carotene + Zeaxanthin (1:1)	--	21	41	0.76	1.9

1/ Chicks were fed the white corn diet with 2% lard for two weeks prior to addition of 8.8 mg of the pigment per kg of diet for a 5-week test period.

2/ F, fed pigment found in skin ($\mu\text{g}/100 \text{ cm}^2$); T, total pigment found in skin ($\mu\text{g}/100 \text{ cm}^2$); C, total pigment consumed (mg/bird).

experiments, (A) and (B), the total amount of pigment consumed per group was approximately the same even though the diets were fed ad libitum. The F/T ratios (F, fed pigment found in skin; T, total pigment found in skin; expressed

as $\mu\text{g}/100\text{ cm}^2$) show that the pigment found in the skin was largely the dietary pigment in each case. However, so little β -carotene was deposited that the pigments found in that group were largely carotenols, probably representing undepleted initial stores plus small accruals from the "white" corn. All carotenols were deposited predominantly in the ester form.

The T/C ratios (T, total pigment found in skin; C, total pigment consumed in mg/bird) provide a means for comparing relative efficiencies of deposition of the different pigments. Since the ratios for zeaxanthin and lutein were essentially the same, one concludes that these two carotenediols are deposited with equal efficiency when fed as the individual pigments. In the comparison between mono-ols, however, cryptoxanthin was at least twice as efficient as the α -ring type, zeinoxanthin. Ratios for β -carotene and the polyoxy fraction were not meaningful because of the poor deposition of the former and the heterogeneity of the latter. Although canthaxanthin was deposited much more efficiently than β -apo-8'-carotenal the skin color produced was unnatural in appearance.

Results for neolutein, the absorption curves of which were the same as the "isolutein" described by Strain, Leaf Xanthophylls, Carnegie Inst., Washington, 1938, indicate that cis-isomerization during processing does not seriously diminish the pigmenting effectiveness. The effects are considerably less damaging than have been observed in the cis-isomerization of provitamin A.

Results with yellow corn. When two selections of yellow corn, one high in lutein, No. 1809 (Oh 45 x B 38) the other high in zeaxanthin, No. 1808 (Lombard, open pollinated), were compared with a commercial yellow corn of unknown pedigree, a difference was observed in pigmenting efficiency. The two corn selections, analyses for which are shown in Table 2, were fed at a level to supply 8.8 mg of carotenols per kg of diet, with and without different levels of lard, for a test period of 4 weeks (Table 3). The commercial corn, which was obtained from the local elevator and was substituted completely for the white corn in the diet, provided about 11.5 mg of carotenols per kg.

Table 2.--Pigment composition of corn selections ($\mu\text{g}/\text{g}$)

Pigment	No. 1808	No. 1809	Commercial
β -Carotene	5.3	6.2	1.0
β -Zeacarotene	2.4	3.6	0.8
ζ -Carotene	0.9	2.7	0.9
Zeinoxanthin	0.6	2.2	0.4
Cryptoxanthin	6.9	2.6	0.9
Esters (as zeaxanthin)	1.2	0.8	0.5
Lutein	5.5	20.6	10.1
Zeaxanthin	27.6	3.7	5.8
Polyoxy pigments	2.1	0.5	0.8

The high-zeaxanthin corn was a hard flinty endosperm type while the high lutein corns were both types with softer endosperm. It is possible that the differences in efficiency ratios, T/C, reflect differences in digestibility and absorption of the carotenols of the endosperm. The type of corn may be an important factor in determining efficiency of utilization of carotenols present. This subject is worthy of further study.

Table 3.--Pigmenting effect of corn selections at different fat levels

Number of birds	Source of pigment	Lard in diet	Dietary pigment a/	Pigment consumed	Pigment in skin	Ratio b/ T/C
		%	mg/kg	mg/bird	µg/100 cm ²	
1 (8)	None	2	0	--	trace	--
2 (6)	Corn 1809	0	8.8	15.2	69.9	4.6
3 (6)	Corn 1809	2	8.8	13.2	75.4	5.7
4 (6) c/	Corn 1809	2	8.8	14.0	70.4	5.0
5 (8)	Corn 1809	10	8.8	13.2	82.8	6.3
6 (6)	Corn 1808	0	8.8	14.8	42.4	2.9
7 (6)	Corn 1808	2	8.8	16.8	56.0	3.3
8 (6)	Commercial corn	2	11.5	20.8	83.8	4.0

a/ Chicks were fed the white corn diet for 2 weeks prior to substitution of yellow corn for a 4-week test period.

b/ See Table 1.

c/ Diet contained 0.017% of added D,L- α -tocopherol.

Alfalfa pigments vs. corn pigments. Alfalfa differs substantially from corn in types and relative quantities of the different xanthophylls present. Lutein and zeaxanthin constitute about 75% of the oxygenated carotenes in most of the dent corn inbreds (Quackenbush *et al.*, *Cereal Chem.* 40:250, 1963) while they make up less than 50% of those in alfalfa (Bickoff *et al.*, *J. Agr. Food Chem.* 2:563, 1964). In this difference may lie the explanation of observations that the xanthophylls of yellow corn are utilized more efficiently than those of alfalfa in skin pigmentation of birds (Day and Williams, *Poultry Sci.* 37:1373, 1958; Ratcliff *et al.*, *Poultry Sci.* 41:1529, 1962). Fritz and Wharton (*Poultry Sci.* 36:1118, Abstr., 1957) also reported that yellow corn produced more skin pigmentation than was expected. Although our data for corn No. 1809 present a similar suggestion, a direct comparison of the T/C ratios of Tables 1 and 3 is not possible because of the difference in length of test period. The absorption of carotenoids by the chick is incomplete at best; consequently, the efficiency of the process is subject to influence by various factors as is evidenced from the response to increased fat in the diet (Table 3).

In recent years, a number of analytical procedures have come into use which specify elution with the mixture of equal volumes of methanol and hexane to obtain "total xanthophylls" in corn and its byproducts and in alfalfa meals. These eluates will include pigments which may have little or no activity such as violaxanthin (Marusich, De Ritter, and Bauernfeind, *Poultry Sci.* 39:1338, 1960) and other polyoxy pigments. Under some conditions, these eluates also include certain amounts of chlorophylls as evidenced from light absorption in the region near 650 m μ as well as in the xanthophyll range. Such results for total xanthophylls are accordingly unrealistically high when related to lutein and zeaxanthin with respect to pigmenting efficiency.

Studies on an analytical method for xanthophylls. About a year ago we proposed use of equal parts of acetone and hexane for the elution of the xanthophylls from a magnesia column in the analysis of the xanthophylls from an alfalfa meal extract. In our hands, this procedure had eluted the major xanthophylls with quantitative retention of the chlorophylls on the column. It was suggested that various laboratories test this procedure. A number

tested it, but some reported elution of the xanthophylls to be slow, perhaps too slow for a satisfactory method. One laboratory (Archer-Daniels-Midland) used the procedure on a large scale as a preparative method for obtaining fractions to be tested in the yolk-pigmentation studies. After elution with equal parts of acetone-hexane, the large column was washed with a mixture of 8 parts methanol to 92 hexane. Mr. Holt kindly sent me portions of the extracts for examination in our laboratory. We examined each of the two fractions to determine the individual pigments present in each (Table 4).

Table 4.--Composition of eluates from large MgO column
Lutein fraction (acetone-hexane, 1:1)

<u>Pigment</u>	<u>%</u>
Carotenes	4.0
Lutein	71.7
Neolutein ("isolutein")	17.1
Unidentified	6.8
Total	99.6

Zeaxanthin fraction (methanol-hexane, 8.92)

<u>Pigment</u>	<u>%</u>
Lutein (and violaxanthin?)	15.0
Zeaxanthin	36.5
Neoxanthin	24.0
Chlorophylls	22.0
Unidentified	2.5
Total	100.0

Undoubtedly a longer period of elution with acetone-hexane would have removed a larger amount of the xanthophylls from the column, since part of the lutein and virtually all of the zeaxanthin remained on the column and these were eluted with the methanol-hexane eluant subsequently. However, acetone-hexane does not elute neoxanthin even with prolonged washing, nor does it elute the chlorophylls. The total pigment eluted by acetone-hexane was much greater in quantity than that subsequently eluted by methanol-hexane.

It is well known that chlorophylls do not contribute to skin or yolk pigmentation. Violaxanthin has also been found of no value in pigmenting egg yolks (Marusich, De Ritter, and Bauernfeind, Poultry Sci. 39:1338, 1960). Whether neoxanthin has appreciable pigmenting effect is uncertain. We presume it is one of the components of the "polyoxy pigments" which we tested in our studies but the amount present in those fractions is not known. While it is evident that 8% methanol gives high results because of the elution of chlorophylls, it is not known to what extent other pigments contribute to this error.

Because of the possibility that neoxanthin may make a contribution to pigmentation, efforts have been made to determine whether this pigment can be separated from the chlorophylls with a smaller amount of methanol. Four percent methanol in hexane will elute neoxanthin. However, this eluant also elutes a substantial amount of chlorophylls (Figure 1). In this figure, the solid line represents a typical absorption curve for neoxanthin as measured

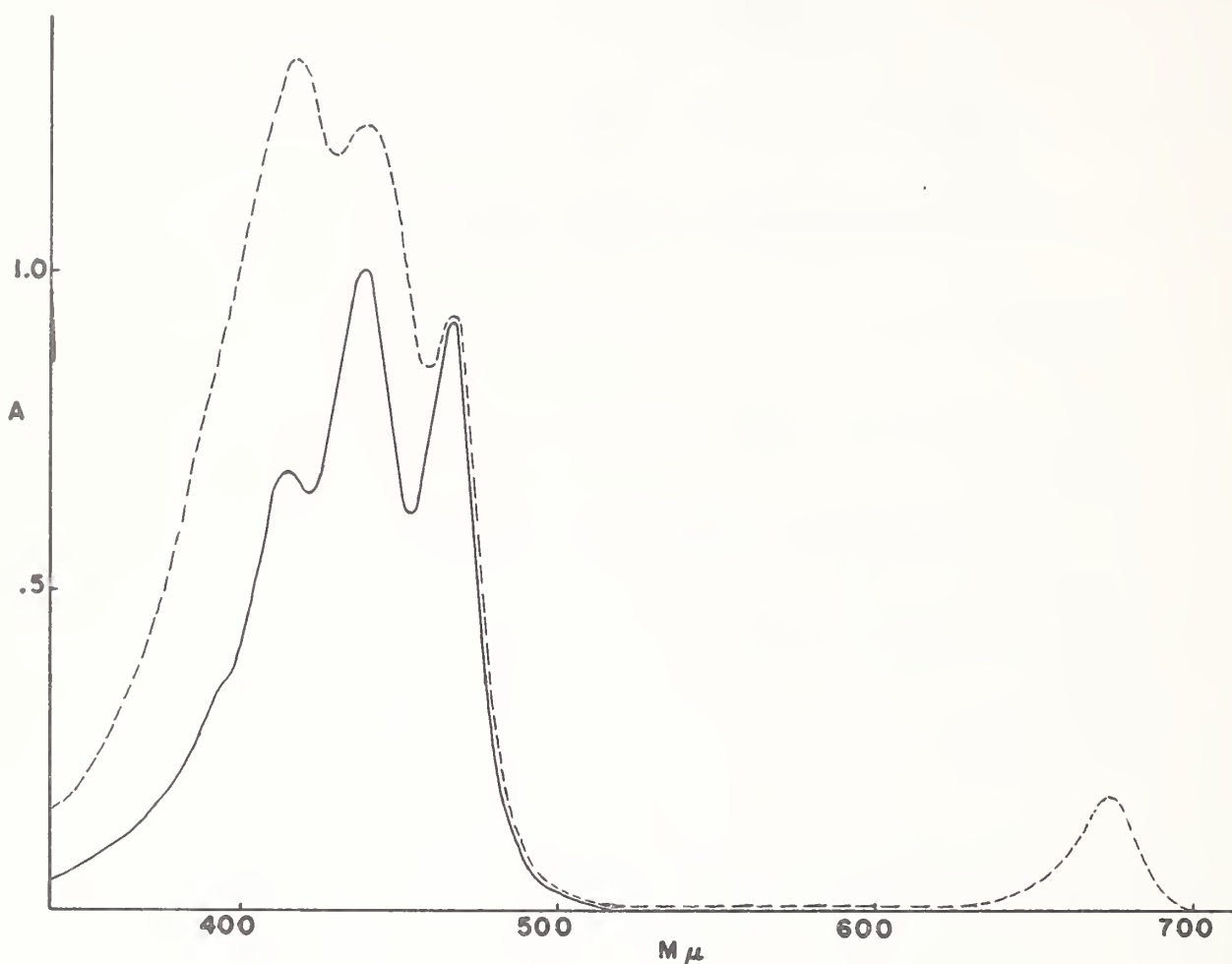


Figure 1. Spectral absorption curves of neoxanthin (solid line) and methanol-hexane (4:96) eluate after acetone-hexane elution (broken line). Absorption at 650-690 mμ is evidence of chlorophyll contamination.

spectrophotometrically. The broken line represents the absorbance of an eluate of 4% methanol in hexane after the column has been washed with equal parts of acetone and hexane. The absorption at 650-690 mμ is typical of the chlorophylls. No carotenoid pigments absorb in this range. However, the chlorophylls also absorb even more strongly in the region of 400-460 mμ, and they are undoubtedly responsible for much of the distortion of the broken line in comparison with the solid line in the figure. Because the chlorophylls absorb in this range where carotenoids are measured they contribute to the total value and give high results. This error can be minimized but not prevented by measuring xanthophylls at 475 mμ.

It is evident, therefore, that if we want realistic values for xanthophylls in alfalfa meal which are representative of pigmenting effect obtainable with the yolks and skin, we must either (a) stop using methanol for elution or (b) remove the chlorophylls before chromatography. Although acetone-hexane leaves a small amount of color on the column, the results are still probably too high when expressed as lutein equivalent because of the low activity of some of the pigments which are eluted. These include cryptoxanthin, zeinoxanthin, and perhaps also violaxanthin and neoxanthin. There is need for experimental data on skin pigmentation of pure samples of the latter two pigments.

Summary. Studies with individual pigments have shown that lutein and zeaxanthin are approximately equal as skin pigmenting agents. Isomerization of the lutein during dehydration of alfalfa does not seriously lower the pigmenting effect. It is apparent that none of the other pigments in alfalfa are equivalent to lutein and zeaxanthin as skin-pigmenting agents for broilers, and that lower values should therefore be assigned to them in an analytical method.

Exclusion of the chlorophylls from the final solution to be measured spectrophotometrically is essential if realistic results are to be obtained in analysis. Accordingly, methanol cannot be used as an eluant unless steps are taken to remove the chlorophylls prior to chromatography.

BIOLOGICAL VALUE OF ALFALFA XANTHOPHYLLS FOR EGG YOLK PIGMENTATION

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Day and Williams reported that the xanthophylls of yellow corn were more efficiently utilized for broiler skin pigmentation than those of corn gluten meal or alfalfa meal (Poultry Sci. 37:1373-81, 1958). Similar results have been reported for egg yolk pigmentation by Morehouse and Hanson (Poultry Sci. 40:1432, 1961) and by Thumin (Seminar on Poultry Husbandry at Herzlia, Israel, 1961, reported by J. C. Fritz at 1962 Fact Finding Conference). Day and Williams (cited above) found BHT to reduce broiler pigmentation somewhat,

whereas 5% beef tallow significantly increased the utilization of xanthophylls. No effect from 4 or 8% corn oil in the diet or from 5% tallow added to dehydrated whole alfalfa or alfalfa leaf meal was obtained in a series of studies conducted at this station (Carlson, 1965, unpublished data). In previous work from our laboratory (Carlson et al., Poultry Sci. 43:654-62, 1964) ethoxyquin at 0.011, 0.015, or 0.25% did not improve utilization of xanthophylls in alfalfa meal for egg yolk pigmentation where the diets were used essentially fresh. On the other hand, Anjaneylu et al. (Poultry Sci. 40:1372, 1961) and Madiedo and Sunde (Poultry Sci. 41:1660, 1962) found ethoxyquin to increase xanthophyll utilization, probably by preventing destruction in the stored feed.

Since there appeared to be a lack of availability of xanthophylls from dehydrated alfalfa meal, studies were undertaken with the aim of improving this situation. The first attempt involved a turbogrinding procedure with the thought that by rupturing the cell walls the xanthophylls would be made more available to the chicken. However, reducing the particle size to 20 μ and thus rupturing the cell walls did not influence xanthophyll utilization (Carlson et al., 1964, cited above). Evidently the cell wall of the feedstuff does not interfere with the hen's ability to absorb xanthophylls.

Further consideration suggested that the chloroplasts had not been damaged by the turbogrinding procedure; therefore perhaps some technique which would disrupt their integrity might be of value. The pelleting process, with heat and shearing forces brought into play, apparently does cause rupture of some of the chloroplasts at least, and this procedure proved beneficial. The results of this work will be described, along with data obtained with super grades of alfalfa meal discussed earlier in this program. In addition, in connection with developments of the chemical assay procedures for xanthophylls, various fractions obtained were tested for their biological pigmentation potency and these results also will be presented.

Experimental. Single comb White Leghorn hens were used in all experiments. For Experiment 3 the hens were housed in floor pens as well as individual cages, whereas for all other studies the hens were kept in cages. Yolk color was scored weekly and visually by the Heiman-Carver (H-C) color rotor (U.S. Egg and Poultry Mag. 41(8):40-41, 1935) and monthly and chemically by the AOAC (9th ed., 1960) method for β -carotene equivalents (BCE). A 16% protein milo-soy diet was used, adequate in all known nutrients. The various dehydrated alfalfa meals studied were added to the diet at a 5% level replacing milo, thus maintaining nearly isonitrogenous conditions.

A concurrent study with Experiment 3 was conducted with broiler-type chicks using some of the same samples of the alfalfa meals used in the hen study. These supplements replaced corn and soybean meal on an isonitrogenous basis. Ten toe web discs (3/16-inch) from each replicate were extracted 48 hr. with 25 ml. acetone and the pigments determined with a Beckman DU spectrophotometer, reading optical density at 445 m μ in 5 cm. cells. Xanthophyll analyses of the alfalfa samples were conducted by a procedure developed at USDA's Western Lab (Kohler et al., Method for the Determination of Xanthophyll, Proc. 9th Tech. Alfalfa Conf., Lincoln, Nebr., 1965), except that the xanthophyll fraction was eluted with Skellysolve B:acetone:methanol (88:10:2).

Results and discussion. For the first experiment, alfalfa leaf meal was compared with regular 17% protein alfalfa meal unpelleted and pelleted and reground. The alfalfa leaf meal supplied about 15 ppm. and the regular alfalfa meal about 10 ppm. of xanthophylls to the diet. The results are shown in Table 1. Note that the basal diet allowed for production of very pale yolks, scoring 6.2 on the H-C rotor and containing 2.4 mcg. of BCE per gram of yolk. The leaf meal markedly enhanced yolk color, intermediate between that obtained with the untreated and pelleted whole meal. The effects of pelleting observed here showed that a practice that the industry had already adopted was markedly beneficial to xanthophyll utilization.

Table 1.--Effect of pelleting and regrinding alfalfa meal
on egg yolk pigmentation--Experiment 1

Treatment	Yolk color ^{1/}	
	H-C ^{2/} rotor	BCE ^{3/} mcg./g.
Basal ^{4/}	6.2	2.4
Leaf meal	13.4	30.9
Alfalfa meal, 5%	12.3	23.2
Alfalfa meal, pelleted and reground	13.8	40.3

^{1/} Average of two month's production of 10 SCWL hens, sampled weekly.

^{2/} Heiman-Carver color rotor, average of 1 day's production/week.

^{3/} β -carotene equivalents, average of 1 day's production/4 weeks.

^{4/} Milo-soy diet, containing in percent, milo 73, 50%-protein-soybean meal 19, dicalcium phosphate 2, limestone 5, salt mix 1/2, and vitamin supplement 1/2. All minerals and vitamins at or in excess of NRC suggested requirements.

The results of the second experiment confirmed those of the first and also gave evidence that the super grades of meal produced by sorting for particle size were indeed superior for egg yolk pigmentation (Table 2). Whereas in the first experiment the value for BCE was essentially doubled by pelleting, in this experiment it was increased by about 1-1/2 times. Two comments in regard to the particle size are pertinent, one that with the smaller particles, more xanthophylls are supplied and yolk color is further enhanced, the other that particle size in itself is not critical. However, pelleting does enhance utilization since less xanthophylls from the pelleted product gave just as good (or even superior) yolk color as that from the super grades of alfalfa meal.

With the information that one pass through the pelleting machine greatly improved xanthophylls utilization, it was decided to determine if repelleting would have any advantage. Samples of commercially dehydrated alfalfa meal and "separation milled" alfalfa leaf meal were retained as such, with portions being pelleted once and ground and another portion pelleted, ground, repelleted, and reground. Analyses of the experimental products for xanthophyll content are shown in Table 3 and the yolk data obtained from feeding the products to laying hens over a 6-month period are shown in Tables 4 and 5.

In Table 4 the visual scores are given and it is evident that repelleting had no advantage for either product. The differences between the control and single pelleted meals were significant, though not as marked as previously. It

Table 2.-- Effect of pelleting and regrinding alfalfa meal on egg pigmentation -- Experiment 2

Treatment	Added xanthophylls ppm.	Yolk color <u>1/</u>	
		H-C rotor	BCE
Basal	0	6.2	5.0
Whole meal, 5%	15	12.9	30.8
Whole meal, pelleted and reground	16	13.9	45.6
Plus 8 mesh	10	12.4	24.0
-8 +12 mesh	19	13.3	35.9
-12 +100 mesh	23	13.3	47.3

1/ Average of two months' production of 10 SCWL hens, analyses and diet as in Experiment 1.

Table 3.--Analysis of products used in Experiment 3

Sample	Protein	Fiber	Carotene	Xanthophylls
	%	%	ppm.	ppm.
Alfalfa leaf			384	438
" , pelleted	29.1	14.2	369	446
" , repelleted			386	465
Whole alfalfa			290	348
" , pelleted	22.6	21.7	279	324
" , repelleted			263	308

Table 4.--Effect of pelleting and regrinding alfalfa meal on yolk H-C color 1/ -- Experiment 3

	Floor <u>2/</u>	Cages <u>3/</u>
Basal <u>4/</u>	--	6.7a <u>5/</u>
Alfalfa leaf, 5%	13.2b <u>6/</u>	13.7d
Alfalfa leaf, pelleted	13.4b	14.1d
Alfalfa leaf, repelleted	--	13.7d
Whole alfalfa, 5%	12.2a	12.8bc
Whole alfalfa, pelleted	12.5a	13.0c
Whole alfalfa, repelleted	--	12.2b
Mean	12.8	13.4b

1/ Average of visual scores from weekly observations of one-day's production.
2/ Two groups of 60 hens. 3/ Two groups of 15 hens. 4/ As in Experiment 1.
5/ Data followed by like subscripts are not significantly different at the 5% level of probability. 6/ Significantly darker (5%) than eggs from floor-housed hens.

is interesting that the caged hens produced darker yolks than floor-housed hens. This was a highly significant difference and was even more evident for the BCE data shown in Table 5. Hens in cages normally must maintain a somewhat larger body size and therefore they show greater feed intake. In this instance, however, a colder environment of the floor-housed hens made for poorer feed efficiency. It thus appears that hens in cages are simply superior in regard to utilization of xanthophylls, and a further explanation for this is in order.

Table 5.--Effect of pelleting of alfalfa meal on egg yolk pigmentation
β-carotene equivalents 1/ -- Experiment 3

	Floor	Cages	Mean
Basal	--	3.9	--
Alfalfa leaf, 5%	38.7	43.4	41.1c <u>2/</u>
Alfalfa leaf, pelleted	39.9	47.2	43.5d
Alfalfa leaf, repelleted	--	46.0	--
Whole alfalfa, 5%	29.0	33.5	31.2a
Whole alfalfa, pelleted	32.2	36.9	34.5b
Whole alfalfa, repelleted	--	32.7	--
Mean	34.9	40.3 <u>3/</u>	

1/ Average of analyses made of one day's production at 4-week intervals over a 6-month period. 2/ Data followed by different subscript are significantly different at the 5% level of probability. 3/ Highly significant difference in BCE of egg yolks from floor housed stock.

The data obtained with these same samples in the broiler pigmentation study are shown in Table 6. Repelleted samples were insufficient for inclusion in this study. However, there is no question but that at each level supplied, the xanthophylls from the pelleted alfalfas were more efficiently utilized than those from untreated alfalfas, especially alfalfa leaf meal. The percentage improvement in pigmentation due to pelleting amounted to 61% for the whole meal and 100% for the leaf meal (see Figure 1).

Table 6.--Effect of pelleting whole alfalfa or leaf meal on
pigmentation in broilers 1/

Alfalfa level, %	Whole	Whole pelleted	Leaf	Leaf pelleted
<u>Optical density of solution from toe plugs</u>				
0	(.195)			
3	.285	.306	.302	.345
6	.337	.374	.358	.424
12	.374	.426	.406	.458
<u>Growth - g. at 4 weeks</u>				
0	668, 670			
3	681	696	697	671
6	681	693	670	655
12	676	684	677	682
<u>Gain/feed ratio</u>				
0	0.71, 0.70			
3	0.69	0.70	0.71	0.70
6	0.69	0.69	0.70	0.69
12	0.68	0.68	0.68	0.68

1/ Same samples as used for hen Experiment 3.

The data for growth suggest that the alfalfa meals were supplying a factor or factors which improved the rate of growth. This could be one of the so-called UGF substances. No effects were observed for feed utilization.

Following these studies, attention was given to comparing pigmentation effectiveness of the various carotenoid fractions obtained in the analysis of alfalfa for xanthophylls. To establish a procedure, a xanthophyll concentrate

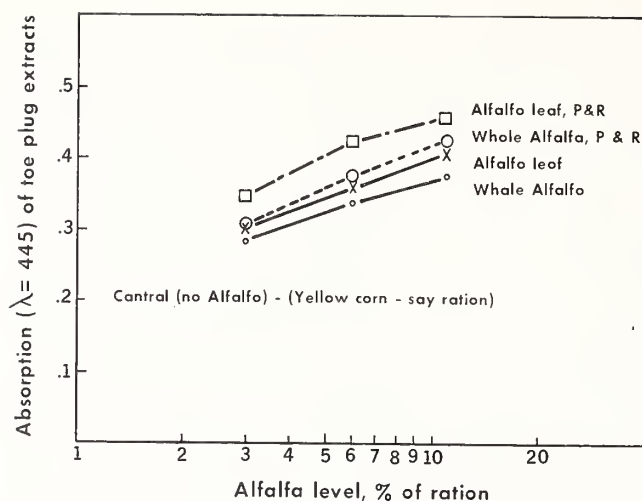


Figure 1. Skin pigmentation of broiler chicks at 4 weeks of age.

from alfalfa--containing 11.3 mg. per ml. of corn oil carrier--was obtained for this work. Hens that had been depleted of xanthophylls by having been on the milo-soy diet for 2 months were individually fed an exact amount of xanthophylls in a gelatin capsule each day. A comparison was made with β' -apo-8'-carotenal, the product described by Marusich *et al.*, 1960. Six hens were given each treatment at each of two levels. The hens were intubated over a 7-day period and all eggs saved from the 3rd day of treatment through 10 days following the last intubation. The yolks were pooled from each hen and a figure for total yield could thus be obtained by analyzing the total yolk mass. The data for this preliminary study are shown in Table 7.

Table 7.--Alfalfa xanthophylls 1/vs. β -apo-8'-carotenal as egg yolk pigmenters -- Experiment 4

Treatment	Level	Yolk BCE <u>2</u> /	Yield <u>3</u> /
	mg/hen/day	mcg./g.	%
Basal	--	2.6	--
Alfalfa xanthophyll	1.13	5.9	1.2
" "	4.52	11.3	1.2
β -apo-8'-carotenal	1.132	15.6	10.1
" "	4.52	31.8	9.0

1/ Given to 6 hens each day over a 7-day period, the alfalfa xanthophylls were obtained by chromatographic separation, using Skellysolve B:acetone: methanol (88:10:2). 2/ Analysis of entire yolk mass obtained during the experimental period and for 12 days following termination of the treatments. 3/ Over and above that obtained in the basal diet.

Essentially the hens received either 1.1 or 4.5 mg. of the xanthophylls per day or a total for the week of 7.7 or 31.5 mg. respectively. The hens on the basal diet were producing yolks averaging about 2.6 mcg. of xanthophylls per g. of yolk. Those receiving the alfalfa xanthophylls produced a yolk mass containing 5.9 or 11.3 mcg./g. respectively. Subtracting that in the yolk from basal-fed hens, calculations were made which showed the yields were very poor--1.2%. On the other hand, the β -apo-8'-carotenal was more efficiently utilized,

to the extent of about 10%. No reason is available to the authors for this difference; a further study on this point would be necessary to elucidate this observation. It doesn't seem likely that an absorption difference of that magnitude exists, based on chemical structure alone.

Subsequently, fractions of alfalfa xanthophylls were obtained and tested in the same manner. The alfalfa extract was saponified and chromatographed on a magnesium oxide: celite column (1:1). The column was washed with Skellysolve B:acetone (9:1) to elute β -carotene, followed by Skellysolve B:acetone:methanol (88:10:2) to elute the dihydroxy fraction and an 80:10:10 mixture to elute a polyoxy fraction.

To show how rapidly the hen puts these pigments into the egg and then how rapidly the pigmentation tapers off, the data in Table 8 are presented. Again, 6 hens per treatment were intubated over a 6-day period in this case with 4 mg. of the respective carotenoids per day. The peak of deposition occurred on the 6th to 9th day after treatment was initiated, with almost complete depletion by 9 days after the treatments were terminated. β -Carotene showed little pigmentation efficiency, in confirmation of many previous studies, and although the bulk of the pigmenting property is in the dihydroxy fraction, there is considerable activity in the polyoxy fraction.

Table 8.--Alfalfa xanthophylls and egg yolk pigmentation -- Experiment 5					
Treatment 1/	β -Carotene equivalents - 3-day periods				
	1	2	3	4	5
β -Carotene	2.6	4.7	4.2	4.7	2.6
Dihydroxy carotenoids	19.8	24.7	16.7	7.8	3.9
Polyoxy carotenoids	10.2	14.1	13.1	4.7	3.6

1/ Four mg. of the material tested given to each of 6 hens each day for 6 days. The data given are the averages for the yolk mass produced in the intervals indicated.

The data are summarized for all observations in Table 9. Note the much improved efficiency of utilization in this study. This is a highly variable determination; many factors affect the efficiency yield calculations, particularly rate of egg production.

Table 9.--Utilization of alfalfa xanthophylls for egg yolk pigmentation -- Experiment 5			
Treatment	Average maximum 1/ H-C score	Average β -carotene 2/ equivalents mcg/g	Yield %
β -carotene	7.4	3.7	.54
Dihydroxy carotenoids	13.0	14.6	5.78
Polyoxy carotenoids	10.6	9.2	3.68

1/ Of daily observations of all eggs produced.

2/ Average of analyses of eggs produced at the 3-day intervals shown in Table 8.

A further study was conducted with these same fractions as well as with a lutein concentrate obtained from Mr. Kenneth Holt of Archer-Daniels-Midland Co., Minneapolis, Minn. This fraction was characterized as containing 71.7% true lutein by Quackenbush (unpublished data, 1965). It had been eluted from the magnesium-celite with a petroleum ether:acetone (50:50) mixture. The data are given in Table 10.

Table 10.--Utilization of alfalfa xanthophylls for egg yolk pigmentation -- Experiment 6

Treatment <u>1</u> /	Average maximum H-C score	Average β -carotene equivalents	Yield %
β -carotene	7.4	2.8	0.16
Dihydroxy carotenoids	15.0	15.4	6.21
Polyoxy carotenoids	10.6	4.5	1.41
Lutein concentrate <u>2</u> /	14.8	14.1	4.95

1/ As in Experiment 5, 4 mg./day for 6 days, given to each of 6 hens/treatment. 2/ Obtained through the courtesy of Mr. Kenneth Holt of Archer-Daniels-Midland Co., Minneapolis, Minn.

In this study the β -carotene and polyoxy fractions were comparatively less efficiently utilized, whereas the dihydroxy fraction was slightly better utilized than before. Essentially there were no real differences between the dihydroxy carotenoid and the lutein fractions.

Summary. In studies using SCWL hens and broiler chicks it was shown that super grades of dehydrated alfalfa meal were more effective as pigmenters than the regular grades. Essentially they contained more initial xanthophylls. One pass of alfalfa meals through a pellet mill enhanced xanthophyll utilization as much as 10% over a 6-month period and by 50 to 100% over a shorter period when measured by chemical assay for β -carotene equivalents. The differences in visual scores obtained by comparison with the Helman-Carver color rotor were not as great, although they were in the same direction. Putting the alfalfa meal through the pellet mill a second time had no value in this regard. The improvement in broiler skin pigmentation by pelleting was in the order of 60% for the whole alfalfa and 100% for the alfalfa leaf meal.

Studies with various carotenoid fractions obtained from alfalfa show that lutein, or the dihydroxy carotenoids, account for the major amount of egg yolk color. However the polyoxy carotenoids do contain pigmentation properties and should not be overlooked in the "xanthophylls" assay.

Acknowledgments. The assistance of Mrs. D. W. White in running analyses on the alfalfas and toe plugs and of Mr. J. W. Nelson in preparing the xanthophyll concentrates is gratefully acknowledged.

AN IMPROVED METHOD FOR THE DETERMINATION OF XANTHOPHYLL

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Since it is not practical for a feed formulator to evaluate the pigmenting potency of alfalfa meal by broiler or egg-yolk assays, it is essential that a chemical method be developed which will accurately measure and predict pigmenting values of samples of dehydrated alfalfa meal.

As mentioned in an earlier presentation at this conference, the xanthophyll picture in dehydrated alfalfa is indeed complex. There are more than 40 yellow components in a typical alfalfa meal (Bickoff et al., J. Agr. Food Chem. 2:563-67, 1954). Apparently any chemical measurement for the xanthophylls will involve a number of compromises.

First of all some published work has demonstrated that not all of the xanthophylls are equally potent as pigmenters for poultry products (Marusich et al., Poultry Sci. 39:1338, 1960). Therefore any chemical method which determines total xanthophylls will have to compensate for these differences. Secondly, the individual xanthophylls differ in their absorptivity and color intensity at various wavelengths of light (Strain, Leaf Xanthophylls, Carnegie Institute of Washington, Wash. D. C., 1938). Therefore, color measurements in a colorimeter or spectrophotometer will have to be made at a "best suitable" wavelength, employing a "best suitable" absorption coefficient.

In our Laboratory in Albany, Calif., we are studying these and other factors in order to develop a satisfactory procedure for the measurement of pigmentation potency. To date we have not completed the evaluation of the pigmenting potency of the individual xanthophylls or their isomers. Accordingly, the method described here represents an interim procedure rather than a final method for measuring potency.

In our present procedure 2-g. samples of alfalfa meal are weighed into 100-ml. volumetric flasks and extracted overnight with 30 ml. of a mixture of Skellysolve B and acetone (7:3). The following day, 1 ml. of a solution of potassium hydroxide in methanol is added and the sample gently mixed by swirling. One milliliter of water is then added and the sample again mixed. During our studies, we investigated several levels of KOH in methanol in order to ascertain the lowest level of KOH which would saponify the chlorophyll. We found that 1 ml. of methanol containing 0.5 g. of KOH would saponify essentially all of the chlorophyll, and, in combination with 1 ml. of water, would enhance the extraction of the carotenoids.

Following the KOH treatment the sample is adjusted to 100 ml. with Skellysolve B and stored in the dark at room temperature for a period of one hour. An aliquot of the sample is then transferred to a separatory funnel and washed with water to remove the methanol, which may interfere with chromatography. After drying over sodium sulfate a portion of the aliquot is added to a chromatographic column packed with activated MgO:Celite (1:1).

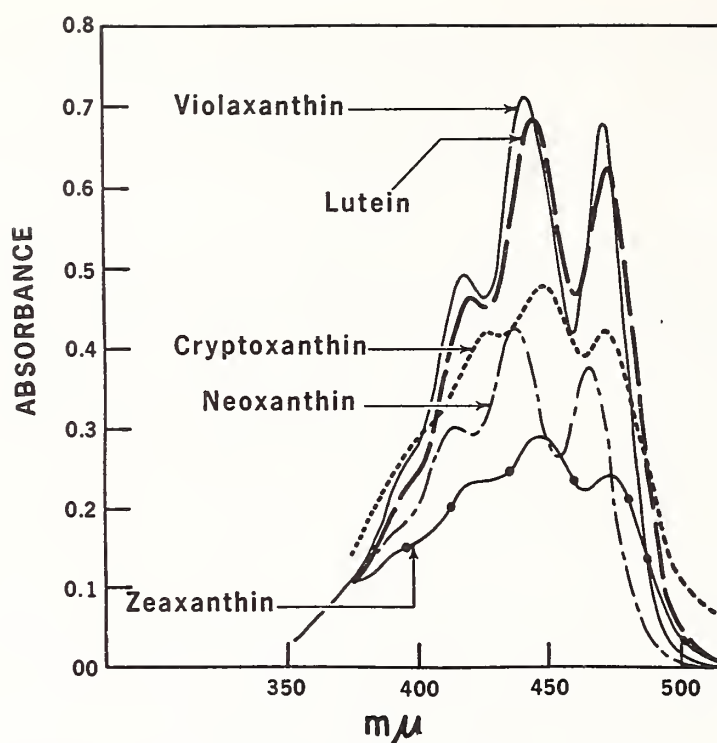


Figure 1. UV absorption spectra for xanthophylls in alfalfa.

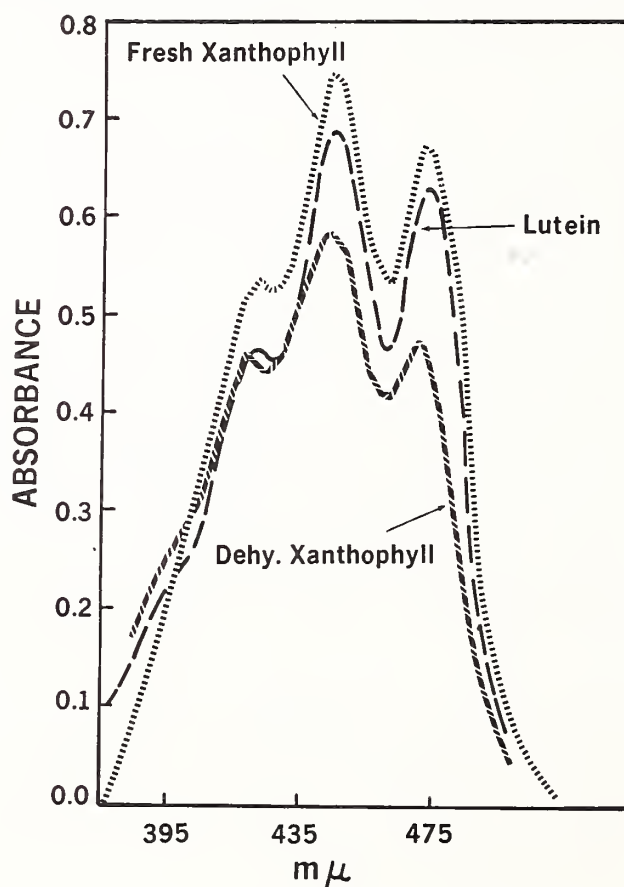


Figure 2. Total xanthophylls in fresh and dehydrated alfalfa.

Acetone:Skellysolve B (10:90) is used to elute the carotenes. At this point the small amount of methanol used in the saponification step may interfere by eluting the cryptoxanthin fraction along with the carotenes.

A mixture of Skellysolve B:acetone:methanol (80:10:10) is used to remove the xanthophylls, following elution of the carotene. We have found that this level of methanol elutes the neoxanthin and polyoxy along with the dihydroxy xanthophylls. If lower levels of methanol are used to elute the xanthophyll fraction, the neoxanthin and polyoxy may be left on the column. For example, we have found that washing of the column with Skellysolve B:acetone:methanol (88:10:2) leaves both of these components on the column, while if Skellysolve B:acetone:methanol (85:10:5) is used as the eluant, the neoxanthin is removed while the polyoxy remains on the column. Until neoxanthin and polyoxy are evaluated as pigmenters, we have included them in the xanthophyll fraction; however, as I will demonstrate in a moment, they are discounted by measuring the xanthophylls at 475 m μ . Both the neoxanthin and polyoxy fractions are made up of several yellow components, which absorb at shorter wavelengths than the principal xanthophyll fraction.

Washing the chromatographic column with lesser levels of methanol may result in leaving some of the other xanthophylls, in addition to neoxanthin and polyoxy, on the column. For example, in a recent cooperative study with Mr. Ken Holt of Archer-Daniels-Midland, Dr. F. W. Quackenbush of Purdue, and Dr. C. W. Carlson of South Dakota, we found that a mixture of acetone:hexane (1:1) eluted lutein and its isomers in addition to cryptoxanthin from the chromatographic column, while further washing with hexane:alcohol (92:8) eluted zeaxanthin, violaxanthin and neoxanthin. Isolation and evaluation of these latter two xanthophylls as pigmenters may establish that they are only partially as potent as lutein. Until this is confirmed, however, we have included them in the proposed procedure with the lutein.

The optical density or absorbance of the xanthophyll fraction is then read on a spectrophotometer or on a standardized colorimeter. At the present we are measuring the absorbance at 475 m μ employing an absorption coefficient of 230. This is approximately at the peak of the second maximum of lutein and employs the lutein absorption coefficient. This is also approximately at the second maximum of three of the other xanthophylls: violaxanthin, zeaxanthin and cryptoxanthin (Figure 1). However, neoxanthin is discounted by about 35% since the maximum of its second peak is at approximately 465 m μ .

One of the remaining problems is that during commercial dehydration and storage of alfalfa, considerable oxidation and isomerization of lutein as well as other xanthophylls occur. This results in the maxima of the mixture moving to the shorter wavelengths and discounts the apparent xanthophyll content (Figure 2).

Another remaining problem before we can chemically assay the pigmenting potency of alfalfa is to isolate the individual xanthophylls and their isomers and evaluate their pigmenting values. Then we can decide which to include in the assay and select the wavelength at which to measure the absorbance of the xanthophylls, and apply the best suited absorption coefficient. We are working on this problem and will report further progress as problems are resolved.

PIGMENTATION POTENCY OF DIFFERENT COMMERCIAL GRADES OF
DEHYDRATED ALFALFA

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In selecting feed ingredients to enhance the pigmentation of broiler carcasses and egg yolks, the feed formulator usually considers several factors: (1) xanthophyll content, (2) cost of the ingredient, (3) level of energy, protein, etc. furnished by the ingredient, and (4) availability of xanthophyll from the ingredient. Very little research has been conducted relative to the comparative availability of hydroxy carotenoids (xanthophylls) from various sources for pigmentation.

Materials and methods. In a recent study at the University of Nebraska we have determined the availability of xanthophylls from ten different sources for egg yolk pigmentation. Four commercial dehydrated alfalfa meals (containing 17, 18, 20, and 22% protein), dehydrated cultivated grass and clover meal, corn gluten meal, florafil, β -apo-8'-carotenal, canthaxanthin, and purified xanthophyll were included in this study. All sources were carefully assayed for xanthophyll content. Xanthophyll analysis data are presented in Table 1. Each source furnished xanthophyll levels of 2.2 and 4.4 mg. per 100 g. (10 and 20 mg. per lb.) in a white corn diet. The composition of this diet is presented in Table 2.

Table 1.--Xanthophyll analyses of feed ingredient sources 1/

Ingredient	Xanthophyll content	
	mg./lb.	mg./kg. (ppm.)
Dehy. alfalfa meal (17% protein)	120	264
Dehy. alfalfa meal (18% protein)	165	363
Dehy. alfalfa meal (20% protein)	181	398
Dehy. alfalfa meal (22% protein)	181	398
Dehy. cultivated grass & clover meal	365	803
Corn gluten meal	36 <u>2/</u>	79
Florafil	3,350	7,370

1/ These values were determined by the Archer-Daniels-Midland Co., Minneapolis, Minn.

2/ The determination for corn gluten meal was apparently in error. This product usually contains 100 mg. of xanthophyll per lb. (200 ppm.). The yolk color value obtained with this source ingredient also confirms a possible error in the analysis.

SC White Leghorn hens were maintained on the white corn basal diet for 90 days prior to the experiment. Six birds with consistent egg production records were assigned to each of 22 treatments. Initially and at weekly intervals throughout the six-week experiment, yolk color was determined following the β -carotene procedure. Determinations were run on two-day-per-week composite egg samples from each treatment.

Results and conclusions. Data pertaining to the effect of ten xanthophyll sources on egg yolk pigmentation are presented in Table 3. Four commercial

Table 2.--Composition of white corn basal diet, 1964--
xanthophyll availability and egg yolk color study

Ingredient	%
Ground white corn	53.8
Wheat standard middlings	1.8 to 20.0
Soybean meal (44% protein)	8.3
Meat and bone scrap	5.0
Fish meal (60% protein)	2.5
Ground limestone	5.1
Defluorinated phosphate	1.0
Iodized salt	0.3
Vitamin premix	1.0
White grease	1.5 to 4.5
<u>Calculated composition:</u>	
Protein, %	16.0
Productive energy, Kcal/lb.	915
Calcium, %	3.0
Total phosphorus, %	0.9
"Available" phosphorus, %	0.6
Methionine and cystine, %	0.54

Table 3.--Effect of ten xanthophyll sources fed at two levels
on egg yolk pigmentation

Xanthophyll source and level	β -carotene equivalents/g. fresh yolk <u>1/</u>	
	10 mg./lb.	20 mg./lb.
Dehy. alfalfa meal (17% protein)	26.0b	57.1cd
" " " (18% protein)	31.4b	44.0bc
" " " (20% protein)	27.4b	51.1bcd
" " " (22% protein)	27.1b	46.8bcd
Dehy. cultivated grass & clover meal	25.1b	44.3bc
Corn gluten meal	81.7d	--
Florafil	25.1b	59.4c
β -apo-8'-carotenal	23.1b	41.4b
Canthaxanthin	46.6c	--
Canthaxanthin & dehy. alfalfa meal	--	58.0cd
Canthaxanthin & corn gluten meal	--	102.0e
Purified xanthophyll	23.7b	45.4bcd
Control (White corn basal diet)	7.1a	8.0a

1/ Each value is the average of seven determinations run at weekly intervals on two-day-per-week composite egg yolk samples. Values followed by the same letter are not significantly different from each other ($P < 0.05$).

dehydrated alfalfa meals (17, 18, 20, and 22% protein), dehydrated cultivated grass and clover meal, florafil, β -apo-8'-carotenal, and purified xanthophyll gave similar yolk color values at both levels of supplementation. Canthaxanthin and corn gluten meal produced significantly ($P < 0.05$) higher yolk color values than other sources. The higher value for corn gluten meal was due to an apparent error in the chemical analysis of this ingredient. Canthaxanthin at 2.2 mg./100 g. of diet (10 mg./lb.) produced an undesirable pinkish-red egg yolk color. Commercial dehydrated alfalfa meals and the other sources produced satisfactory egg yolk color or pigmentation.

THE ALFALFA INDUSTRY VIEWPOINT ON XANTHOPHYLL AND PIGMENTATION

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The concern of the dehydrated alfalfa industry for xanthophyll and xanthophyll assay procedures has been one of short duration. In early 1962 some processors started the practice of guaranteeing xanthophyll content of dehydrated alfalfa meal. Immediately it became apparent that a standard assay method was needed if the industry was to take full market advantage of the xanthophyll in alfalfa meal.

In late 1963 the Executive Committee of ADA appointed a Technical Committee of industry personnel. It directed this new Committee to work with consumer and government laboratories to develop a xanthophyll method that could be used in the trading of dehydrated alfalfa meal and recommended to AOAC for adoption as an official procedure.

The newly formed Technical Committee held its initial meeting in February of 1964. At that meeting seven xanthophyll assay procedures employed by processors and customers were presented to the Committee for consideration. Before the Committee could start evaluating these methods, it was faced with a major decision. Should all xanthophyll pigments be measured or should only one or two of the xanthophylls known to have high pigmenting properties be assayed? The following decision was reached by the Committee: "It was agreed that a uniform method would have to measure all xanthophylls until such a time as it has been definitely proved that some do not have pigmenting biological activity for poultry. Any other course of action would have a most serious marketing repercussion, would have to be matched by similar partial measurement for other pigmenting ingredients, and could be compared to measuring separately or a selected group of various amino acids rather than total protein."

The experimental work being done in laboratories of the men appearing in this conference is bringing us closer to an identification of those xanthophylls that have pigmenting characteristics; however, much work must be done before the pigmenting properties of each of the xanthophylls has been determined and these answers translated into a method that can be run routinely in a processor or consumer control laboratory. Until this work has been completed I believe the viewpoint of the alfalfa industry is summed up in the paragraph that I read from the minutes of the first Technical Committee meeting.

Four of the seven methods were chosen by the Committee for a collaborative study with each of the methods selected representing a different technique. The methods were identified as Quackenbush, ADM, Schenck, and Kohler. Dr. Thomas Sullivan was preparing to conduct a study, at the University of Nebraska, on the effect of various xanthophyll sources on egg yolk color and the Technical Committee decided to correlate their collaborative work with Dr. Sullivan's study by assaying samples of the materials he planned to feed. This included four alfalfa meals of varying protein content, dehydrated grass and clover meal, corn gluten meal, florafil, and yellow corn meal. A set of samples was to be analyzed at the beginning of the feeding study and again at the end. Each laboratory was requested to analyze each sample twice using each of the four

methods selected. The final color readings were to be made on a Beckman DU at 447 mμ and 475 mμ or a Colorimeter if a Beckman DU was not available. These instruments were to be standardized with a standard xanthophyll furnished by Dr. Schenck. In addition to the Technical Committee laboratories, a number of laboratories representing state, federal and consumer interests participated in the collaborative program.

When the results of these analyses were completed and tabulated, it was apparent that the development of a suitable xanthophyll method was much further away than the Committee had originally assumed. The results were submitted to a statistician who returned them stating that the data were so bad there would be no value in running a statistical analysis. The data and experiences of the analysts with the methods were discussed at a meeting of the collaborators last December. It was agreed that further collaborative study should be delayed until more information is available on the pigmenting characteristics of the xanthophylls eluted by methods under study.

These poor results should have been anticipated by the Committee since the results of the ADA and AAFCO Carotene Check Sample have been equally bad during the many years in which it has been conducted. Most of you are familiar with these check sample reports and the inability of the participating laboratories to check within a reasonable limit. The average standard deviation for the four samples sent out in 1964 was 7.9 with the between-laboratory 95% confidence limit of 21.9.

The Technical Committee decided to submit a questionnaire to all of the carotene check sample participants to determine whether these laboratories were following the official AOAC Method 39.015 and, if not, whether specific variations in technique could account for the differences in results obtained. Sixty of the check-sample participants completed and returned the questionnaire and a study of the answers showed that no two participants are running the carotene analysis exactly the same. An attempt was made to correlate certain differences in techniques with high and low results reported on the check samples; however, there were so many interrelating variations that such a comparison was impossible. A tabulation of the answers received on the questionnaires was made and distributed to the participants with the hope that the laboratories would change their procedures to conform to the official AOAC method. There are, however, many areas in which the official method should be more specific and there is a need for review and revision of this method.

It is not possible for me to review the entire questionnaire here, but I have selected a few of the items that may be causing discrepancies in our carotene analysis. Sixteen brands of acetone are being used, including five specification grades ranging from industrial to analytical reagent. Fifteen brands of petroleum ether are used with nine boiling point ranges as purchasing specifications. Three types of magnesia are purchased from nine suppliers with 40% of the laboratories reporting that their magnesia is over one year old. The chromatographic columns range from a 10-mm. ID to over 22 mm. ID and are loaded with magnesia from under 6 cm. to over 12 cm. in height.

Sixty-five percent of the laboratories grind the sample; the rest do not. Sixty percent use a sample of 1 g. or under; the remainder use 2 g. or more.

Approximately half the laboratories extract 16 hr. overnight at room temperature; the remainder extract from 1 to 3 hr. on a hot plate. About 50% of the participants filter the extract before elution and 45% use an aliquot of the extract for eluting. Twelve types of spectrophotometers and colorimeters are used for reading the eluted carotene; only half of the participants indicated that they standardized their instruments, using seven different methods for standardization.

When one considers all of the liberties that our analysts have taken with this method, it is amazing that the results of the check samples are as good as they are. I point out the discrepancies of our carotene analysis since the carotene must be eluted prior to the xanthophyll fraction and if our laboratories cannot extract, elute, and measure the carotene with accuracy and precision, we certainly cannot expect that these laboratories will analyze the xanthophyll accurately regardless of the excellence of the method developed. I have been associated with analysts long enough to know that each believes the changes which he has made in a method give the most accurate results; however, when a method is used for trading purposes or referee work, it is essential that each laboratory adhere to the method published.

The ADA Technical Committee and AOAC collaborators recognized that the procedure for carotene and subsequent xanthophyll analysis does need study and revision and some of the changes being considered are: (1) Establishing tighter specifications for the acetone and petroleum ether reagents. (2) Removal of chlorophyll prior to chromatographing. (3) Establishing a central supply of magnesia of guaranteed quality. (4) Setting up a uniform procedure for standardizing spectrophotometers and colorimeters. We hope that these and other improvements can soon be incorporated into the carotene method and proposed xanthophyll method.

To summarize: The dehydrated alfalfa industry needs methods for carotene and xanthophyll that will produce accurate and precise results in the hands of our routine control analysts. The methods must be fast and utilize a minimum of reagents and equipment. The assay for xanthophyll must include all xanthophylls that have pigmenting characteristics. This is a big order and I am not sure that it can be accomplished; however, because the men who have appeared on the program today are working on the problem, I believe the possibilities are very good. Once the methods have been developed and adopted, each of us must insist that his laboratory runs the methods as written. Otherwise, the work done by this group will be in vain.

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